ALTERNATIVE PRACTICES IN ORGANIC DAIRY AND BROILER PRODUCTION AND THEIR EFFECTS ON ANIMAL BEHAVIOR, HEALTH, AND WELFARE

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HANNAH NICOLE PHILLIPS

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BRADLEY J. HEINS

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Chapter 1: Background

1.1. Organic Livestock Production

The history of organic agriculture provides insight to the core values of today's organic livestock industry. Agriculture became polarized in the United States (**US**) at the turn of the Environmental Revolution in the 1970's over concerns about chemical fertilizers and pesticides [1]. After years of organic industry groups requesting the protection of their farming practices, congress passed the Organic Foods Production Act of 1990, which required that the United States Department of Agriculture (**USDA**) create national standards for all aspects of organic agriculture to help unify the multiplicity of practices. In 2001, the USDA created the National Organic Program (**NOP**) and Code of Federal Regulations (Title 7, Subtitle B, Chapter I, Subchapter M, Part 205)[1] to protect the integrity of the organic seal and mandate regulations. For example, all organic farms must undergo a certifying process by an NOP accredited agency. Although there are several technical differences between organic and conventional livestock systems,[2] the major defining characteristics include

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[1] This part of the Code of Federal Regulations will be referenced as § 205 henceforth.

[2] The term *conventional* is an ambiguous term used to describe non-organic systems. However, there are some cases where conventional farms may adopt some organic practices, such as grazing and alternative therapies. Henceforth, *conventional* is defined as "non-organic livestock systems that keep animals in total indoor confinement and have the ability to utilize treatments when necessary that are unallowed in organic practices, such as antibiotics."

grazing and outdoor access requirements and prohibition of most synthetic substances (e.g., antibiotics).[3]

The National Organic Standards Board (**NOSB**) — a Federal Advisory Board comprised of 15 volunteers — reviews current standards and reports recommendations to the NOP. For example, the NOSB may review and recommend the allowance of certain synthetic substances if a justified need exists, and evidence supports its safety to people and the environment. Another example of the NOSB's responsibilities includes their efforts to amend pasture space requirements for poultry. If the NOP accepts the NOSB recommendations, the NOP initiates rulemaking to change The National List of Allowed and Prohibited Substances (§ 205.607) in the Code of Federal Regulations, which is available to the public.

Today's organic industry is one of the fastest growing agricultural segments in the world [2]. In the US, the organic livestock sector is dominated by the dairy and poultry industries [3,4]. The top reported reasons why organic dairy producers choose to transition from conventional systems are to: 1) avoid chemicals and pesticides, 2) enhance economic viability and 3) improve the environment and soil [5]. These explanations expose modern motivations, yet reported themes still honor the earliest organic values of fostering natural

[3] *Organic production* is defined by the NOP as "a production system that is managed in accordance with the [Organic Foods Production Act of 1990] and regulations in this part to respond to site-specific conditions by integrating cultural, biological, and mechanical practices that foster cycling of resources, promote ecological balance, and conserve biodiversity" (§ 205.2).

systems. Organic livestock producers respect and promote a natural environment for their animals, which happens to be an important component of animal welfare.

1.2. Animal Welfare

Animal welfare is multifactorial; all components of an animal's life contribute to their overall wellbeing [6]. There are several definitions of animal welfare, such as Broom's 1986 definition — "the welfare of an individual is its state as regards its attempts to cope with its environment" — [7], The Five Freedoms developed between 1965 and 1979 [8,9], the Five Domains developed in 1994 [10], and The Allostasis Concept that appeared in 2007 [11]. Although all definitions contribute to the understanding of animal welfare, the Fraser et al. [12] framework best describes how the organic industry values animal welfare.

In 1997, Fraser et al. [12] developed a holistic framework consisting of three overlapping ethical concerns in which animal welfare can be evaluated and human preference can be categorized (Fig 1.1). The framework's ethical views are: 1) animals should function well in terms of satisfactory health and physiology (i.e., biological function), 2) animals should experience natural lives (i.e., natural living) and 3) animals should be free of intense and prolonged negative emotional states (i.e., affective state). When evaluating animal welfare, people tend to emphasize the importance of one category over the others. For example, the NOP dairy standards value systems that mimic nature and commend practices that maximize the natural lives of animals — the natural living component of the animal welfare framework. Thus, strong valuation for natural living is prevalent among many organic producers [13].

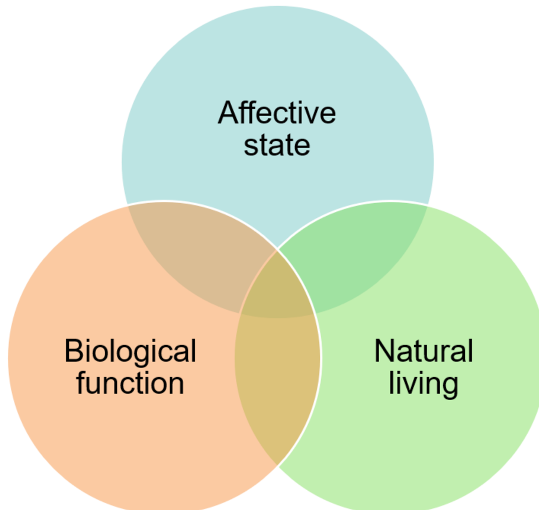

Figure 1.1. The animal welfare framework. Descriptions were developed by Fraser et al. [12].

Organic standards emphasize that animals should live as naturally as possible, which is generally accomplished by requiring animals to be reared with access to the outdoors, restricted periods of indoor confinement, and reduced stocking densities [14]. Animals raised organically may have more freedom to express natural behaviors compared to animals living in conventional systems. For example, Gonçalves et al. [15] reported that access to outdoors for broilers can improve the expression of natural behaviors, such as dust and sunbathing. Weeks et al. [16] found that broilers with access to the outdoors ran and foraged (i.e., pecked at the ground) more than broilers raised in total confinement and fed a high-density diet. In another example, Sanchez-Casanova et al. [17] showed that outdoor access reduced corticosterone in young broilers that were 4 weeks old, indicating reduced stress. Furthermore, access to the outdoors may have a positive effect on animal health in specific cases. In a review of literature on behavioral differences between cows housed with and without pasture access, Charlton and Rutter [18] suggested that the pasture environment may alleviate some animal health issues that are aggravated in total indoor confinement systems, such as lameness and hock lesions possibly caused by exposure to hard (e.g., concrete) flooring and resting areas. Alternatively, the pasture environment can introduce other challenges that may jeopardize animal welfare, such as biting flies [19] and heat stress [20,21] in dairy cows, and predation in chickens [22]. Animals living in organic systems may have some advantages for improved animal welfare compared to those raised in conventional systems, especially in terms of abilities to perform natural behaviors and alleviate animal welfare issues exacerbated by total confinement. Yet, the pasture environment presents its own animal welfare challenges, and there are several other facets of organic practices to consider that potentially affect animal welfare.

Placing most of the focus on the natural living component of animal welfare may be problematic for organic animals because emphasis in only one category may come at the expense of the others. To support this idea, previous literature acknowledged the deficits in organic livestock production regarding the biological function and affective state categories [23–25]. For example, Bergman et al. [26] found that organic dairy farms were less compliant compared to their conventional counterparts on the use of pain relief for disbudding calves, which may be partially due to the limited organic-approved options of pain relief. In a survey on bovine veterinarians' perspectives on organic livestock production, Sorge et al. [27] found that many veterinarians disagreed that animal health was better on organic farms and expressed concern that the lack of proven effective therapies may impair animal welfare. Furthermore, the surveyed veterinarians reported that they often struggled to successfully treat sick animals with alternative therapies within the regulatory framework of NOP [27]. It is evident that there are many disadvantages to organic animal production systems, especially when animals need a treatment intervention and alternative therapies fail.

It is noteworthy to acknowledge that animals have preferences within their living environment. Previous studies found that dairy cows have a partial preference for pasture, which is contingent on many factors, including time of day, weather and individual variation [28–30]. In studies of broiler chickens, Taylor et al. [31,32] found evidence that outdoor ranging varies between individuals within the same flock, indicating partial preference for ranging depending on the individual. It seems intuitive to think that animals raised in organic systems — where the freedom of choice is allowed — have better welfare, though the opportunity for choice may not necessarily relate to improved animal welfare, as animals may not consistently choose what is in the best interest of their welfare.

Motivation tests have been used to determine how hard an animal is willing to work to gain access to a resource [33]. It has been suggested that strong motivation to access a resource indicates that the resource is important to the animal, and denying that resource has a negative effect on animal welfare [34,35]. In an experiment by von Keyserlingk et al. [36], dairy cows were trained to push open a weighted gate to access either fresh feed or pasture. Cows pushed a similar weight to access fresh feed and pasture but pushed more weight to gain pasture access at night [36]. Another experiment by Charlton et al. [29] reported that dairy cows' time on pasture declined when walking distance increased during the day, but distance did not influence nighttime pasture use. Therefore, pasture access may be an especially important resource for dairy cows at night. Individual variation may also play a major role in preferences. For example, Ferreira et al. [37] found that broilers with high ranging levels preferred to "work" for their food (i.e., pecking through straw, wood shavings, and hemp litter for mealworms) whereas broilers with low ranging levels preferred food that was readily available (i.e., mealworms presented without a foraging substrate), suggesting that an exploratory trait may be a driver for motivation to access resources in broilers. Therefore, the ability of an animal to access a resource that is highly important may influence animal welfare, but further research needs to verify whether having this access directly improves animal welfare.

There is currently no strong evidence on whether animals reared in organic systems have inferior or superior animal welfare compared with animals in conventional systems [38]. Furthermore, the level of animal welfare is likely contingent on various management factors and complex situations. For example, Sutherland et al. [25] reported that mastitis is the most common and important health issue for dairy cows regardless of organic status. While mastitis may be less common on some organic dairy farms [39,40], antibiotics are prohibited in organic production so the ability to effectively treat organic cows for mastitis is limited. Ruegg [41] reported that alternative therapies, such as whey-based therapies, garlic tincture and aloe vera are commonly used to treat mastitis in organic cows, but limited research exists on whether these therapies are actually effective. Promoting animal welfare is a challenging balancing act between the three overlapping ethical concerns. Identifying animal welfare deficits in organic livestock production is the first step in capitalizing on these opportunities to improve welfare.

For the remainder of this introduction, several areas for research will be highlighted with respect to gaps in organic production for animal welfare. These topics include alternative pain management therapies for disbudding dairy calves, distress and mastitis prevention in transitioning dairy heifers, face fly larva control using broilers, and outdoor stocking densities for free-range broilers. These topics were decided based on current trends and interests in the organic dairy and broiler industries. Furthermore, lack of scientific evidence on these topics makes them of high priority for research.

1.3. Pain Management for Disbudding

1.3.1. Horn Removal

Whether performed under conventional or organic management, horn removal is a major animal welfare concern among industry and public stakeholders [42,43]. However, the majority of dairy producers in the US (94%) remove horns [44]. Horns are perceived as a risk for animal and human injury and are therefore undesirable [45]. Yet, there is little evidence showing that horns are a risk for animal or human injury [46]. In fact, it has been suggested that horn removal has little benefit to animal and human safety [47]. At the present, there is evidence of farmer, veterinarian, researcher and citizen stakeholder interest in preserving horns [42,48]. In the US, there are no known current studies on horned dairy cattle, so it is difficult to accurately enumerate the presence of horned organic herds. In the European Union, a survey of 419 dairy farms estimated that 78% of organic farms maintained horns [49]. Perhaps unaccounted horned organic dairy herds exist in the US, especially considering current trends in the European Union. Preserving horns as a strategy to enhance dairy cattle welfare is insufficiently investigated and represents a research topic of high priority. However, horn removal practices are still dominant in the organic dairy industry [5,26], thus scientific investigation on ways to mitigate pain inflicted by horn removal procedures remains necessary.

Dehorning is the most painful and least desired method of horn removal [50]. Therefore, the dairy industry has advocated for farmers to disbud calves instead [51,52]. *Dehorning* is defined as "the process of removing the horn of an adult cow after the horn has developed attachment to the skull", while *disbudding* is defined as "the process of damaging the horn bud tissue in young calves to prevent the growth of horns" [50]. Over the years, disbudding has increased in popularity as a method of horn removal, such that disbudding was practiced on 86% of dairy farms in 2014 the US [44]. The two major methods used to disbud calves include cauterization and caustic paste [44]; however, caustic paste is generally not approved for organic use since it contains chemicals that destroy the horn bud tissue after topical application (§ 205.603). Furthermore, the use of caustic paste can be problematic since it has been demonstrated in clinical trials to cause pain and can become dangerous if accidently transferred to other parts of the body [53,54]. Therefore, caustic paste should be promoted with caution since it could encourage farmers to rear calves in isolation, which has detrimental effects on animal welfare [55]. Cautery disbudding represents the major method for horn removal in organic dairy calves and a widespread animal welfare issue for the organic sector.

Pain is the most significant acute effect of cautery disbudding. Calves exhibit intense and frequent escape behaviors during the procedure [56], and elevated pain and wound sensitivities for at least 24 hours after the procedure [57,58]. Stewart et al. [59] showed deviations in ocular temperature within minutes after disbudding, suggesting immediate pain following disbudding. Neave et al. [60] discovered that calves were less likely to perform an ambiguous task at 6 and 22 hours after disbudding, suggesting “pessimism” in disbudded calves. Recent studies even suggest that disbudded calves experience prolonged pain before [61] and after [62] the wounds re-epithelialize, which take approximately 9 weeks to heal [61]. The long-term pain of disbudding is poorly understood and could have ramifications on the welfare of adult cows. Studies in rats found that early-life pain trauma [63] and stress trauma [64] increased adulthood stress- and anxiety-specific responses in novel environments. Furthermore, another study suggests that early-life distress related to isolation in dairy calves has negative effects on immediate development and future behavior, cognition and coping strategies [55]. Therefore, disbudding is a major animal welfare concern with potential long-term negative effects and strategies to minimize pain should be utilized. The NOP recommends implementing practices which minimize acute pain and stress caused by the disbudding procedure using effective methods and approved therapies. However, organic producers have limited pain mitigation therapy options (§ 205.238), making disbudding pain management a challenge and widespread animal welfare issue for the organic sector.

1.3.2. Pain Management

The best way to alleviate acute disbudding pain is through multimodal therapy, using multiple methods to manage pain. In a review of 21 studies by Winder et al. [65], it was suggested that the combination of a cornual nerve block with a local anesthetic and a systemic non-steroidal anti-inflammatory drug

(**NSAID**) increases acute analgesia compared to a local anesthetic alone. The local anesthetic induces a localized insensitivity in the horn bud area and the NSAID systemically reduces inflammation by inhibiting the enzyme cyclooxygenase (**COX**) and consequent synthesis of inflammatory prostaglandins, such as prostaglandin E_2 (**PGE2**) [66]. This multimodal method is useful because local anesthetics have a functional duration of approximately 90 minutes [67], and a long-lasting NSAID may alleviate the inflammatory pain thereafter [65].

The use of disbudding pain alleviation methods on organic farms is quite low and depends on several factors of feasibility. A recent survey of 189 US organic dairy producers reported that less than 26% of farms used either a local anesthetic or an NSAID for disbudding calves [26], and the use of multimodal pain therapy is estimated to be rare [68]. Organic producers are restricted to substances that are approved by the NOP (§ 205.603) and the few NSAID options available limits the feasibility of proper pain alleviation. For example, lidocaine (a local anesthetic) and aspirin (an NSAID) were added to The National List of Allowed and Prohibited Substances in 1995 and are generally acknowledged as substances that accommodate organic values [70,71]. However, aspirin is not approved by the Food and Drug Administration (**FDA**) for use in cattle and is therefore not allowed. In 2016, the withdraw period for lidocaine was reduced from 90 to 8 days for animals intended for slaughter and from 7 to 6 days for animals intended for dairy [71]. In 2007, flunixin (an NSAID) was added to The National List of Allowed and Prohibited Substances in favor of its positive impact on animal welfare [72]. However, flunixin was simultaneously strongly opposed by farmers and NOSB reviewers who were charged by its contradiction of organic values [72]. Furthermore, flunixin must be administered intravenously (**i.v.**), which may be a contributing factor to its lack of adoption since i.v. methods may be challenging and unappealing to some producers [73]. Consequently, organic farmers have demonstrated reluctance to implement flunixin as a post-operative pain management therapy, but have expressed interest in plant-based alternatives to alleviate pain [40].

Organic dairy producer and veterinary stakeholders have either adopted or exhibited an interest in non-synthetic substances, such as herbal therapies, to mitigate disbudding pain [26,40]. A survey of over 180 US organic dairy farms reported that 21% used a naturally derived therapy as pain management for horn removal procedures [26]. However, these alternative therapies may be a problematic solution since their efficacy is mostly based on anecdotal evidence. Previous surveys of over 290 organic dairy producers and veterinarians in the US similarly recognized the deficit in knowledge about effective organic-approved practices [74,75]. Furthermore, the use of ineffective alternative practices — and consequent prolonged suffering — has been identified as a major threat to organic dairy animal welfare. In a review of dairy industry changes that affect animal welfare, Barkema et al. [76] proposed that future research should focus on identifying effective organic-approved alternative remedies.

1.3.3. Research Methods

To shed scientific light on current alternative therapies used in organic livestock production, the aim of Chapter 2 is to address whether a commonly used alternative pain mitigation therapy product can be used as a replacement for the current predominant method (i.e., lidocaine) for disbudding. The methodology for evaluating disbudding pain and stress in Chapter 2 relies on the best described approaches for measuring disbudding pain and stress in calves: blood cortisol and behavior.

Pain and stress are challenging to quantify and understand in animals. Physiological measures of pain can be useful but also require careful interpretation. The body responds to pain by triggering an autonomic nervous system (**ANS**) response [77]. In particular, the sympathetic nervous system (**SNS**) of the ANS orchestrates a fight-or-flight response, in which the brain communicates to the adrenal gland via converging systems; the sympathetic-adrenal-medullary (**SAM**) system uses electrical signals and the hypothalamic–pituitary–adrenal (**HPA**) axis uses a series of cascading hormones to prompt the adrenal gland [77]. The SAM system quickly triggers the adrenal gland to release catecholamines, such as adrenaline and norepinephrine, to increase vigilance and ultimately prepare the body for immediate physical reaction [77]. The HPA axis stimulates the adrenal gland to release glucocorticoids, such as cortisol, which have a variety of prolonged functions, including immune and inflammatory suppression [78]. Therefore, pain and stress of animals can be inferred by observing elevated hormones involved in the SAM and HPA axis function [78]. However, the HPA axis hormones may be problematic measurements of pain since they also elevate in response to other categories of stressors. Furthermore, cortisol plays a positive role in inflammation resolution and healing promotion and so requires careful interpretation when examining pain in subjects with tissue damage [58]. Yet, cortisol remains a staple measurement to interpret acute disbudding pain and preserves the comparability and reproducibility of studies [65].

Quantifying pain-specific behaviors that increase in frequency after disbudding (e.g., ear flicks and head rubs) is another useful tool to draw conclusions about pain in disbudded calves [79]. Although behavior measures may be inconsistent between studies, subjective, time-consuming and variable between individual animals [65,73], it is important to examine diverse pain characteristics in examinations of disbudding pain in calves. Therefore, methods to quantify pain in Chapter 2 will include measurements of behaviors.

1.4. Alternative Non-Steroidal Anti-Inflammatory Drugs

1.4.1. Synthetic Salicylates

Synthetic salicylates, such as acetylsalicylic acid (i.e., aspirin) and sodium salicylate, have previously been used as effective anti-inflammatories, antipyretics (i.e., fever reducers), and analgesics in cattle. In an experiment by Coetzee et al. [80], intravenous sodium salicylate administered at a dose of 50 mg/kg reduced cortisol concentrations when compared to untreated cattle following castration. However, an oral dose of aspirin at 50 mg/kg did not mitigate the cortisol response [80]. In another experiment, Baldridge et al. [81] reported that sodium salicylate dissolved in *ab libitum* drinking water at rates of 2.5 to 5.0 mg/mL and offered from 1 day before to 2 days after castration and dehorning improved average daily gains (**ADG**) for the next 13 days and decreased cortisol concentrations for up to 6 hours following the procedures compared to calves who received no treatment. Although synthetic salicylates show promising utility for pain mitigation in cattle, they have never been officially approved by the FDA and are therefore not permitted.

1.4.2. White Willow Bark

White willow (*Salix alba* L.) bark (**WWB**) is one of the most popular plant-based therapies used for pain relief in humans [82]. As with all plants from the *Salix* genus, white willow bark contains salicylate compounds primarily comprised of salicin [83], which is converted into salicylic acid (**SA**) when consumed orally [84]. Salicylic acid is similar to synthetic salicylates, such that it inhibits the enzyme COX and blocks inflammatory prostaglandins such as PGE2 [85,86]. Various studies reported reductions in pain when administering WWB to humans [87–89]. However, there are currently no studies indicating the usefulness of WWB for alleviating disbudding pain in calves.

White willow bark may be a useful alternative to synthetic salicylates to mitigate the delayed onset of pain in disbudded organic calves. Plant matter, especially leaf and branch trimmings, from the *Salix* genus have been previously demonstrated to be a nutritious feed source in agroforestry systems and safe for consumption by ruminants [90–94], but the efficacy of WWB as an alternative therapy for the purpose of alleviating pain in cattle is currently unsupported by scientific evidence. Furthermore, use of unproven alternative therapies by the organic dairy industry is commonly raised as a major animal welfare concern [13,23,25,26]. Therefore, it is essential that scientific research addresses this exposed knowledge gap by investigating WWB for its analgesic effects in calves.

1.4.3. Research Methods

Therefore, the objectives of Chapter 3 are to: 1) determine the salicin concentration of non-standardized products containing WWB that are currently used or may be used for disbudding pain in organic dairy calves and 2) determine if WWB affects blood concentrations of SA and inflammation in healthy dairy calves.

Salicin is the most prominent compound in WWB extracts responsible for anti-inflammatory effects [95]. However, the amount of salicin in WWB products is not commonly provided by manufacturers. In an observational study to determine the amount of salicin in the bark of various *Salix* species grown in Lithuania, Kenstavičiene et al. [96] found that WWB had 1.21 to 1.87% salicin. In other studies, Pitta et al. [92] and McWilliam et al. [93] found that leaf and branch trimmings from *Salix* species contained 0.09 to 0.17% salicin. High-performance liquid chromatography (**HPLC**) is the most common method to determine the concentration of salicin in plant matter [92,93,96]. Therefore, the salicin concentration of several WWB products that are currently used or may be used by the organic dairy industry to mitigate pain will be evaluated using HPLC.

After ingesting, salicin is converted to several different metabolites from the salicylate family, which can be detected in the plasma of blood. Salicylic acid is the major metabolite that makes up total salicylates detected in the plasma after ingesting salicin. In a pharmacokinetic experiment of oral WWB in humans, salicylic acid made up 86% of the total detected salicylates in the blood serum [97]. The minimum total salicylate plasma concentration needed for analgesia in calves was previously estimated to be 25 to 30 μg/mL [80,98]. Since SA makes up an estimated 86% of total salicylates in the plasma after ingesting salicin [97], the estimated minimum SA plasma concentration needed for analgesia in calves is approximately 21.5 to 25.8 μg/mL. Therefore, plasma concentrations of SA will be measured in calves receiving WWB to determine if the minimum SA plasma concentration needed for analgesia in calves is met and to corroborate inflammation findings.

Non-steroidal anti-inflammatory drugs prevent inflammation by inhibiting COX, the class of enzyme involved in the production of inflammatory prostaglandins [99]. Prostaglandin E_2 is the most notable inflammatory prostaglandin for having the greatest impact on processing of pain signals [100]. There are two main types of COX enzymes: COX-1 and COX-2. Prostaglandins related to COX-1 mostly control homeostatic processes and are involved in the resolution of inflammation, but not the progression of inflammation [101]. Prostaglandins related to COX-2 are mostly associated with pain and inflammation that result from tissue injury [101]. Few studies investigate the specific mechanisms of WWB on COX enzymes. In one study [102], white willow bark inhibited COX-2-mediated PGE2 release *in vitro*. In an investigation of aspirin and salicylate, which have similar mechanisms to salicin, Higgs et al. [103] showed that both NSAIDS mediated PGE2. Furthermore, prostaglandin E_2 has been commonly used as a measurement of inflammation in cattle in numerous previous studies [104,105]. Therefore, prostaglandin E_2 will be measured in calves to understand the effects of WWB on inflammation.

1.6. Managing Transition Heifer Behaviors and Udder Health

1.6.1. Challenges for Early-Lactation Heifers

First-calf heifers encounter several challenges following calving that can jeopardize animal welfare. Firstly, some heifers may become distressed when they encounter unfamiliar experiences related to being milked, such as unfamiliar sounds and smells in the milking parlor and tactile stimulation to the udder by handlers and milking units. For example, Van Reenen et al. [106] found that peak plasma cortisol concentrations were approximately 20% greater for heifers during milking on day 2 compared to day 130 of lactation,[4] indicating that the beginning

[4] The typical lactation period is approximately 305 days [307], so 130 DIM represents mid-lactation.

of the lactation period can be stressful. Sutherland and Huddart [107] also found similar results, in which heifers had 2.0 times the plasma cortisol concentration on the first day in milk (**DIM**) compared to the fifth DIM. Furthermore, authors also reported that plasma oxytocin[5] concentrations after milking preparation procedures (but before milking unit attachment) were 2.4 times greater for heifers at 130 DIM compared to 2 DIM, indicating that heifers may need time to acclimate to milking procedures [106]. It is apparent that the early-lactation period can be distressing for heifers, but it can also be stressful for handlers and certain heifer behaviors may even put heifers at risk for mastitis.

Distressed heifers can endanger the safety of human handlers, as heifers may kick off milking clusters, kick at handlers and display other undesirable behaviors that interfere with milking productivity. This increases the chance of injury to handlers and the risk of mastitis[6] for the animal [106,108–110]. For example, a prospective evaluation of all injuries by cattle at a hospital in New Zealand over a 1-year period conducted by Watts and Meisel [111] showed that hand and wrist injuries were common and tended to occur after being kicked by a cow at milking time. In terms of udder health, Nitz et al. [108] found that heifers that detached milking cups during milking were 2.6 times more likely to develop

[5] *Oxytocin* is defined by the National Mastitis Council as “the hormone produced in the pituitary gland that causes milk let-down” [308].

[6] *Mastitis* is defined by the National Mastitis Council as “inflammation of the udder, most commonly caused by an infecting microorganism” [308].

new intramammary infections (**IMI**)[7] between 3 and 17 DIM. In a cross-sectional study of 46 Swiss farms, Ivemeyer et al. [110] found that the number of kicks per cow displayed during milking was associated with new IMI infection incidences. Therefore, unwanted heifer behaviors during the early-lactation period may jeopardize both human and animal welfare.

In general, heifers are vulnerable to clinical mastitis[8] and IMI during early lactation [112–114]. In an observational study of 1,014 heifers in Sweden, Persson Waller et al. [114] reported that approximately 50% of the 364 recorded clinical mastitis cases in heifers occurred within the first 6 DIM, which were primarily diagnosed with *Staphylococcus aureus*. This is a concern for farmers since poor udder health in heifers is associated with production, treatment, and labor costs. In 2009, Huijps et al. [115] estimated that the costs of clinical mastitis and IMI were $18.75 and $6.56 per heifer, respectively.[9] In a more recent study in 2014, Cha et al. [116] estimated that the average cost of a clinical mastitis case ranged between $115 and $476 after considering mortality and reduced conception costs. Furthermore, poor udder health in early lactation may also put heifers at risk of future infections [117,118]. Negative affective states and poor milking behavior may also represent an economic loss due to risk of IMI [108],

[7] *Intramammary infection* is defined as “the presence of an organism in the udder that is isolated from a milk sample” [309].

[8] *Clinical mastitis* is defined by the National Mastitis Council as “udder inflammation characterized by visible abnormalities in the udder or milk” [308].

[9] Euros were converted to the US dollar based on the average 2009 euro dollar exchange rate of 1.39 (https://www.macrotrends.net/2548/euro-dollar-exchange-rate-historical-chart).

decreased milk productivity [119] and the risk of early culling [109]. Therefore, strategies to prevent distress and mastitis in heifers is of major interest.

1.6.2. Methods to Modulate Distress Behaviors and Mastitis

Several approaches have been considered to reduce distress and prevent mastitis in heifers. In general, these strategies include handling heifers and familiarizing them with the milking parlor before calving [109,120–125]. For example, Hemsworth et al. [109] found that heifers that were handled during calving displayed 40% less flinch, step and kick responses during milking over the first 20 DIM compared to heifers that were not handled during calving. Bertenshaw et al. [121] found that spending 30 to 245 minutes brushing heifers during the last 6 weeks of gestation reduced kicking during milking up to the first 28 DIM compared to heifers that were not brushed. Das and Das [124] found that 30 udder massage sessions lasting 20 minutes each during the last 2 months of gestation improved temperament, milk letdown and milk flow rates over the first 16 DIM. Eicher et al. [125] reported that heifers that moved through the parlor (but not milked) with lactating cows twice daily for 3 weeks prior to calving balked for a shorter amount of time while entering milking stalls on the first DIM compared to heifers that did not receive any treatment prior to calving. However, behaviors of shifting, stomping, kicking and kicking the milking unit off during milking were similar among treatments on the first DIM [125]. Kutzer et al. [123] reported that pre-parturient acclimation, which consisted of introducing heifers to the milking herd at least 10 days before calving and at least 10 visits to the parlor where milking staff provided tactile contact to the udder on the milking platform, reduced post-parturient stepping, kicking, ear flattening, tail tucking and eye widening behaviors in heifers over the first 7 DIM. However, intensive protocols to acclimate heifers to milking procedures such as the ones described in these previous studies may not be feasible for many farms due to labor restraints, so developing a simpler protocol that fits within the capabilities of dairy farms is necessary.

A variety of strategies implemented during the pre-parturient period have been explored to prevent clinical mastitis and IMI, such as internal teat sealants [113,126–128], antibiotic therapies [129–133], milking [125,134,135] and repeated applications of teat dip or spray [136–138]. However, some of these strategies, such as internal teat sealants and antibiotics, are not allowed for use in organic dairy animals in the US. In one experiment by Santos et al. [134], pre-parturient milking 3 times daily for 15 days prior to calving decreased the proportion of heifers with positive bacterial milk cultures by 25% on the first DIM and decreased the incidence of mastitis by 57% during the first 135 DIM. In another experiment, Lopez-Benavides et al. [137] reported that pre-parturient teat spraying with an iodine-based disinfectant 3 times weekly for 21 days prior to calving reduced *Streptococcus uberis* in milk samples immediately after calving by 53% but did not reduce the incidence of clinical mastitis. Yet, labor limitations may prevent the application of these intensive strategies on many farms. Therefore, current pre-parturient strategies for preventing early-lactation clinical mastitis and IMI in heifers need to be improved to be practicable on farms in terms of labor limitations.

1.6.3. Research Methods

The aim of Chapter 4 is to determine whether training consisting of weekly pre-parturient teat dipping in the milking parlor can modulate behavioral responses and decrease clinical mastitis and IMI in heifers over the first 3 DIM. This experiment's training protocol is a simplified version of those that were previously examined, representing a strategy that may fit within the labor restrictions on most farms. The pre-parturient training has two purposes: 1) to modulate post-parturient aversive behaviors and 2) to decrease post-parturient clinical mastitis and IMI.

Aversive behaviors are behaviors that are undesirable to human handlers. These include behaviors that endanger handler safety and behaviors that interfere with milking efficiency. Commonly examined aversive milking behaviors include stomping, kicking and kicking the milking unit off [106,109,123,125,139,140]. Ease of milking parlor entry is also important, as aversive behaviors such as balking may interfere with milking efficacy [125]. Furthermore, objective temperament scores are commonly used to describe the overall reactivity of cows to stressors related to milking procedures [124,141].

Aversive behaviors may also be indicative of distress in heifers. For example, Hemsworth et al. [109] reported that milk cortisol concentrations were associated with flinch, step and kick responses in heifers, indicating that these behaviors may be indicative of distress. Furthermore, Fogsgaard et al. [142] found that cows with mastitis were more restless during milking, indicated by greater frequencies of tripping and kicking, suggesting that the presence of these behaviors may indicate pain caused by mastitis. Therefore, this experiment will assess behavioral reactivity by examining the presence of commonly explored behaviors during milking procedures, including stomping, kicking, kicking the milking unit off, and parlor entry and milking temperament scores.

In general, mastitis can be classified into clinical mastitis (i.e., abnormal milk), subclinical mastitis (i.e., elevated somatic cell count), and/or IMI (i.e., bacteria in the milk) [143]. For the experiment in Chapter 4, mastitis will be assessed by examining clinical mastitis and IMI. The incidence of clinical mastitis is a commonly examined outcome for mastitis in previous studies [126,128,144–146]. Furthermore, clinical mastitis is assessed by staff prior to each milking per normal standard operating procedures, making it a feasible measurement on working farms. Intramammary infections will be assessed by analyzing quarter-level milk samples for the presence of bacteriological pathogens immediately following the final collection of colostrum to represent the initial IMI prevalence in quarters after calving. Bacteria species and level of infection will be evaluated for each milk sample. The methods that will be used to assess the initial IMI prevalence in heifers after calving are similar to previous studies and will help evaluate the effects of pre-partum factors on IMI [108,126,128,144,147].

1.7. Face Fly Management on Dairy Farms

1.7.1. Face Fly Pests

On organic dairy farms in the US and Europe, the face fly (*Musca autumnalis* De Geer) is a common pest of pastured cattle. Adults of the face fly feed primarily on excretions around their host's eyes, and are notorious vectors for the bacterium *Moraxella bovis*, which causes infectious bovine keratoconjunctivitis — commonly known as pinkeye [148]. Cattle attacked by face flies may cope by bunching in a group with their heads toward the center of the group [149], and increasing the rate of head throws as the number of face flies increase [19]. Because face flies harm cattle, suppressing numbers of this pest may improve cattle welfare.

The use of synthetic substances for fly control on organic cattle is restricted by the NOP (§ 205.603). The inclusion of multiple effective fly control methods (i.e., integrated pest management) is an important approach for managing dung breeding flies on organic dairy farms. For example, plant-derived topical products may repel horn flies (*Haematobia irritans* L.) on cattle for up to 3 days after application [150,151], and modern walk-through systems that trap to kill adult horn flies may reduce the number of horn flies on cattle by 44 to 75% [152,153]. Some previous studies suggest that face flies may also be repelled with plant-derived repellents [154]; yet, effective methods for the control of face flies on organic cattle are not well documented.

1.7.2. Chickens and Dung Fly Larvae

Source reduction of face flies with free ranging chickens may be a feasible option for organic dairy farms. Adult face flies lay their eggs in fresh cow manure, which provides nutrition and safety for the development of immatures [155]. Once the larvae mature, they burrow under the dung pat to pupate and finish developing into adult flies. The development of an egg into an adult can take as little as 7 days in the heat of summer or up to 28 days under cooler conditions [155]. Strategies that biologically disrupt the developing larvae could ultimately reduce the proportion of larvae that reach adulthood and may represent a feasible method of pest management on organic dairy farms.

Some producers believe that chickens will consume face fly larvae in dung pats, and that grazing cattle and chickens in succession as part of a diversified system is an effective method of disrupting face fly developments [156]. In a survey conducted in 2012, Sorge et al. [157] reported that 9% of organic dairy farms in Minnesota used foraging chickens as a method of controlling dung flies. In a survey of 18 farmers in California, seventeen percent reported a pest control benefit after adopting pastured poultry practices [158]. However, using chickens as a method of controlling face flies has yet to be evaluated under experimental conditions. The high protein content and digestibility of fly larvae make them a potentially excellent addition to the chicken diet [159], and previous research indicates that the diet of chickens with access to pasture may consist of up to 9% insects on a dry matter basis [160]. However, no scientific studies have determined whether chickens can successfully reduce the survival rate of face fly larvae in cow dung pats on pasture.

1.7.3. Research Methods

The aims of Chapter 5 are thus to: 1) evaluate whether broiler chickens can affect the survival of face fly larvae in cow manure pats and 2) evaluate broiler behaviors to supplement larvae survival findings. The methodology for evaluating larvae survival uses a novel approach since this is the first known experiment to evaluate larvae survival in a field setting. Some methods of a previous study by Valiela [161] are similar to the ones used in Chapter 5, in which manure substrate is inoculated with a known number of larvae, incubated until the larvae pupate and then examined by counting the remaining pupae. A simple calculation yields the survival rate of the larvae:

$$\frac{Number\ of\ remaining\ pupae}{Number\ of\ starting\ larvae} \times 100 = \%\ survival$$

Therefore, the survival rate of larvae in this experiment will be used to evaluate whether exposure to broilers had any effect on larvae survival.

Foraging and ranging outside the coop are necessary behaviors for the successful utilization of broilers as a method of controlling dung fly immature development in this experiment. Similar to previous studies [162–164], the proportion of broilers observed outside over the course of the experimental period will be used to enumerate the level of ranging. Furthermore, foraging, sitting, standing, sleeping, and traveling behaviors will be evaluated by observing focal birds during behavioral observations throughout the experimental period. This method is similar to those described in previous studies [15,164], but will utilize focal sampling instead of scan sampling, which may provide a more detailed description of behaviors compare to scan sampling [165]. Furthermore, undesirable weather conditions may affect broiler behaviors. For example, Stadig et al. [166] found that ambient temperature, rainfall, solar radiation and wind speed affected ranging behaviors in broilers. Therefore, weather conditions will be simultaneously recorded during each behavioral observation.

1.8. Outdoor Stocking Density for Broilers

1.8.1. Organic Regulations

Access to the outdoors is one of the main defining features of organic broiler chicken production systems. Providing broilers free access to the outdoors is intended to improve their wellbeing by allowing them opportunities to express their natural behaviors and mitigate discomfort associated with confinement to indoor housing. Yet, organic standards are vague regarding what “outdoor access” really means:

The producer of an organic livestock operation must establish and maintain year-round livestock living conditions which accommodate the health and natural behavior of animals, including: Year-round access for all animals to the outdoors, shade, shelter, exercise areas, fresh air, clean water for drinking, and direct sunlight, suitable to the species, its stage of life, the climate, and the environment… (§ 205.239 Part A1).

Interpretations of "outdoor access" are wildly inconsistent, ranging between pop holes leading to a small barren space to doors opening to a massive, lush pasture. The certifying agent is responsible for determining if the outdoor area is adequate by evaluating each of the organic standard's criteria (e.g., shade, shelter, exercise areas, fresh air, clean drinking water and direct sunlight). The outdoor access standard for organic broilers has historically been a controversial topic, dividing farming practices and creating discrepancies within the organic sector.

In April 2016, the USDA announced a proposed rule[10] to clarify organic regulations related to animal welfare standards [167]. The proposed rule was based on the NOSB recommendation established in 2011, which notably set standards for outdoor space requirements for organic poultry among defining

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[10] The legislative details of the proposed rule can be found at https://www.federalregister.gov/documents/2016/04/13/2016-08023/national-organic-program-organic-livestock-and-poultry-practices.

other vague livestock standards. In January 2017 — after three delays — the USDA published the Organic Livestock and Poultry Practices (**OLPP**) final rule[11] and sought public comments on whether it should be withdrawn [168]. Among the many amendments contained in the OLPP final rule [168], one of the revisions addressed how much outdoor space is required for broilers:

> *For broilers (Gallus gallus), outdoor space must be provided at a rate of no less than one square foot for every 5.0 pounds of bird in the flock [up to 0.25 m^2 per bird depending on weight]. (p. 7091; § 205.241 Part C6).*

The vast majority (approximately 88%) of over 72,000 commenters expressed their opposition of withdrawing the OLPP final rule while less than 1% supported the withdraw [169]. Despite overwhelming support, the USDA withdrew the OLPP final rule[12] in March 2018 based on their lack of statutory authority and to maintain consistency with their regulatory policy principles [169]. In the proposal for withdrawing the OLPP final rule [169], it was stated that:

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[11] The legislative details of the OLPP final rule can be found at https://www.federalregister.gov/documents/2017/01/19/2017-00888/national-organic-program-nop-organic-livestock-and-poultry-practices.

[12] The legislative details of the OLPP final ruling withdrawal can be found at https://www.federalregister.gov/documents/2018/03/13/2018-05029/national-organic-program-nop-organic-livestock-and-poultry-practices.

> *The relevant language and context … [suggests] … that Organic Foods Production Act's reference … to additional regulatory standards 'for the care' of organically produced livestock does not encompass stand-alone animal welfare concerns, but rather is limited to practices that are similar to those specified by Congress in the statute and necessary to meet congressional objectives… (p. 10776; Section 5 Part A1).*

The USDA's rationale for withdrawing the OLPP final rule was essentially based on the belief that the Organic Foods Production Act lacks the authority for implementing the animal welfare provisions contained in the OLPP final rule.

The OLPP final rule was the first USDA regulation to address animal welfare under NOP organic management, amending vague standards to promote the three overlapping facets of animal welfare by Fraser et al. [12]. One of the major defining features of the OLPP final rule involved defining outdoor space allowances for poultry since the existing regulations were — and still are — incredibly vague. The lack of clear organic livestock practice standards continues to be an issue for the sector, which has caused consumer uncertainty about organic poultry production practices.

1.8.2. Welfare Certification Regulations

Certification programs, such as Certified Humane (Humane Farm Animal Care, Middleburg, VA), American Humane Certified (American Humane, Washington, DC) and Animal Welfare Approved (A Greener World, Terrebonne, OR), have been successful at addressing animal welfare concerns and gaining the trust of consumers. For example, a 2016 survey of 1,000 US consumers of meat, egg, and dairy showed that 70% were willing to pay at least an extra $1.00 per pound of chicken breast if it came from chickens whose welfare was verified under a trustworthy welfare certification [170]. Welfare certification programs rely on the collaboration of animal scientists, veterinarians, and producers to develop standards for humane farming and continually review new information pertaining to improving the lives of farm animals. Many of these programs address how much outdoor space is needed for broilers. Some certifications, such as Animal Welfare Approved [171], have a single outdoor space requirement for all broilers under the certification:

> *After the brooding period each bird must have continuous access to at least 4 sq ft (0.37 sq m) range and foraging area. (p. 15; Section 7 Part 3.9).*

While others, like Certified Humane [172], offer additional “Free-Range” and “Pasture-Raised” labels for broilers that have access to the outdoors:

> *For free-range systems, the minimum outdoor space requirement is 2 square feet per bird [0.19 m^2 per bird]. For pasture-raised systems, the minimum outdoor space requirement is 2.5 acres (1 hectare)/1000 birds [10.1 m^2 per bird]. (p.10; Section 3, Part G29 c–d).*

Although it is implied that more outdoor space is associated with improved broiler welfare, as shown for indoor spaces [173], there is little evidence to support this claim. Previous studies of broilers reared in total confinement have demonstrated that more crowded conditions may reduce leg and footpad health, increase fearfulness, and decrease locomotion and preening behaviors [17,173,174]. In one study by Jones et al. [175], outdoor stocking densities of 1.2 *vs* 2.5 m^2 per bird had no effect on free-range broiler growth, pasture ranging nor behaviors (i.e., drinking, foraging, lying, sleeping, standing and walking), suggesting that the level of outdoor space may not indicate better animal welfare. Yet, the amount of outdoor space remains a major defining characteristic of differing levels of animal welfare certifications. The evidence regarding the effects of outdoor stocking density on broiler welfare is currently an area that meriting investigation.

1.8.3. Research Methods

To build upon regulations of the withdrawn OLPP final rule for organic livestock and animal welfare certification programs, the aim of Chapter 6 is to address whether additional outdoor space affects the welfare of broilers. The methodology for evaluating animal welfare in Chapter 6 excludes approaches that assess biological functioning outcomes, such as tibia characteristics and footpad and leg lesions, commonly applied in experiments to assess levels of crowded indoor stocking densities (e.g., 0.06 to 0.13 m^2 per bird) [174]. Instead, animal welfare is assessed from a natural living perspective, as this facet of animal welfare is emphasized in organic livestock production [13]. Previous studies have used broiler behaviors, such as standing, sitting, sleeping, locomotion, foraging and aggression to understand how indoor stocking density affects animal welfare [176,177]. Studies on free-range broilers include evaluations of range use [163,164,166,178]. Therefore, Chapter 6 compares the effects of two outdoor stocking densities on broiler welfare from a natural living standpoint.

Chapter 2: Evaluation of An Herbal Therapy to Alleviate Acute Pain and Stress of Disbudded Dairy Calves Under Organic Management

2.1. Summary

The objective of this experiment was to evaluate an herbal therapy used in place of standard synthetic analgesia to mitigate disbudding pain of dairy calves. For this experiment, fifty-four calves were randomly assigned to one of three treatments: 1) local anesthetic lidocaine given as a cornual nerve block before cautery disbudding (AD); 2) sham disbudding (SD); or 3) herbal tincture (Dull It, Dr. Paul's Lab, Mazomanie, WI) composed of white willow (*Salix alba* L.) bark, St. John's wort (*Hypericum perforatum* L.), chamomile (*Matricaria recutita* L.), arnica (*Arnica montana* L.) and fennel (*Foeniculum vulgare* Mill.) administered orally before and after cautery disbudding (TD). Behaviors were assessed during disbudding, and behaviors and blood plasma cortisol concentrations were assessed following disbudding. Tail wag, head movement, forcing ahead and kick rates recorded during disbudding were similar among treatments. When averaged across the 360-minute observation period following disbudding, injury-directed behavioral rates of head jerks, head shakes, horn bud scratches and head rubs were greater ($p \leq 0.03$) for calves in the AD group than calves in the SD group, calves in the TD group had greater ($p < 0.01$) horn bud scratch and head rub rates compared to calves in the SD group and calves in the AD group had a greater ($p < 0.01$) horn bud scratch rate than calves in the TD group. Calves in the AD group took 1.6 (95% CI = 1.0 to 2.4, $p = 0.03$) times longer to lie down after disbudding compared to calves in the TD group. Serum cortisol concentrations were greater ($p \leq 0.01$) for calves in the TD group compared to calves in the SD group at 10, 30 and 90 minutes after disbudding. At 30 minutes after disbudding, calves in the AD group had 5.8 ng/mL (95% CI = −1.1 to 12.7 ng/mL, $p = 0.02$) greater serum cortisol compared to calves in the SD group; while calves in the TD group had 14.3 ng/mL (95% CI = 1.5 to 27.1 ng/mL, $p < 0.01$) greater serum cortisol than calves in the AD group. In conclusion, neither the local anesthetic lidocaine nor the orally administered herbal tincture attenuated both acute injury-directed behaviors and blood plasma cortisol concentrations in disbudded calves, and the tincture was clearly less effective at mitigating cortisol; therefore, additional analgesic may be required to properly manage disbudding pain effectively.

Keywords: behavior, calf, cortisol, disbud, herbal medicine, pain

2.2. Introduction

Cautery horn bud removal (i.e., disbudding) of young calves is a common, yet painful procedure practiced on dairy farms. Pain inflicted during the cautery disbudding procedure has been previously verified by using quantitative behavioral measurements, including rates of head movements, tail wags and vocalizations [56,179,180]. Acute pain following disbudding has been documented in numerous previous studies by evaluating blood plasma/serum cortisol concentrations and behaviors focused around the horn bud wounds, such as ear flicks, head rubs and head shakes [56,79,180–184]. Pain following disbudding has also been previously assessed by evaluating a range of behaviors, including lying/standing, maintenance behaviors and rumination [56,79,179,182].

Organic dairy producers have limited analgesic options for mitigating pain in dairy calves undergoing cautery disbudding. In the US, the use of synthetic therapies for mitigating disbudding pain in organic dairy calves is restricted by regulations set forth by the United States Department of Agriculture (USDA) National Organic Program (NOP), which maintains official federal standards for organic production practices. Lidocaine is a commonly used synthetic substance that is approved for use in organic-certified calves and alleviates disbudding pain by providing local analgesia. Lidocaine induces a localized insensitivity in the horn bud area within 2 to 5 minutes and has a functional duration of approximately 90 minutes [185]. Previous studies agree that lidocaine is effective at reducing escape and struggle behaviors during disbudding, acute injury-directed behaviors up to 2 hours after disbudding and acute blood plasma/serum cortisol concentrations up to 1.5 to 3 hours after disbudding [56,179,180]. However, the injection and restraint required for administering lidocaine potentially may cause pain and stress for calves [186], and the use of lidocaine prior to disbudding may prolong pain following the procedure [180,182]. As a possible response to these shortfalls, an emerging interest in non-synthetic alternatives for reducing disbudding pain in organic calves currently exists. In general, organic producers are familiar with using naturally derived therapies, such as herbal-based products for the treatment of mastitis in dairy cows [40]. A survey of over 189 organic dairy farms in the US reported that 21% used a naturally derived therapy as pain management for horn removal procedures as opposed to synthetic therapies [26]. Naturally derived products — which must first be approved by the farm's NOP accredited agency — may represent potential analgesic options for mitigating cautery disbudding pain in organic dairy calves, but this hypothesis must first be evaluated under experimental conditions.

Research on the efficacy of alternative therapies used in organic livestock production is needed to verify that their use indeed improves animal welfare. Disbudding represents a major animal welfare concern among industry and non-industry stakeholders due to the pain the procedure inflicts [42,43]. Previous surveys of over 290 organic dairy producers and veterinarians in the US recognized that the deficit in knowledge about effective organic-approved practices jeopardizes animal welfare [74,75]. Thus, the use of ineffective alternative practices represents a major threat to organic dairy animal welfare. In a review of dairy industry changes that affect animal welfare, Barkema et al. [76] proposed that future research should focus on identifying effective organic-approved alternative remedies.

The objective of this experiment was to evaluate pain-associated behaviors and cortisol concentration of dairy calves that received either an experimental herbal tincture prior to cautery disbudding, the current standard local anesthetic procedures prior to cautery disbudding or no treatment prior to sham disbudding. The hypothesis of this experiment was that calves receiving a local anesthetic before disbudding, an herbal tincture before disbudding or sham disbudding with no treatment would differ in their pain responses during and after hot-iron disbudding.

2.3. Materials and Methods

2.3.1. Animal Housing and Care

The University of Minnesota Institutional Animal Care and Use Committee approved all animal care and procedures specific to this experiment (protocol number 1508-32864A). This experiment was conducted at the University of Minnesota West Central Research and Outreach Center in Morris, MN from May to July 2016 using 54 pre-weaned female calves aged from 35 to 57 days (mean ± SD = 44 ± 1 days). This age range represented the approximate national average for age at disbudding on dairy operations in the US [44]. Calves used in this experiment were either pure Holstein or a crossbreed, as described by Heins et al. [187]. Calves were housed in groups of 10 in straw-bedded pens consisting of a three-sided shelter (3.7 × 6.1 m) with an equal-sized outdoor area. Calves were fed once daily in quantities of 6 L per calf of unprocessed organic whole milk at 08:00, as described by Kienitz et al. [188].

Beginning 10 days prior to the experiment, calves were acclimated to halter restraint and human handling by increasing their exposure to experimental conditions incrementally each day from 30 minutes on the first day to 8 hours on the last day. During the acclimation period, handlers would periodically visit calves to touch their horn buds and neck. The pens were scheduled for disbudding on separate days when the youngest calf in the pen reached 5 weeks of age and when precipitation was not anticipated. After calves were offered milk on the days of the acclimation period and on the day of the experiment, calves were secured to the perimeter fence of the outdoor portion of the pen using a halter and lead. Each calf had enough lead (0.9 m) to lie down, stand up, drink *ab libitum* water from a 3.8 L bucket fastened to the fence and interact with adjacent calves that were 1.5 m apart.

2.3.2. Catheter Placement

Catheters were placed into the jugular vein of calves 24 hours prior to disbudding. While calves were restrained in a chute equipped with a head lock (Caf-Cart, Raytec, Ephrata, PA), hair was clipped around the horn bud area and in a 12-cm band around the neck. The area of catheter placement was surgically prepared with alternating povidone-iodine and 70% isopropyl alcohol scrubs. The hypodermis of the surrounding catheter site was anesthetized by infiltrating 2 mL of 2% lidocaine (Vedco, Saint Joseph, MO). The jugular was punctured with a 14-gauge × 133-mm peripheral venous catheter (BD Angiocath, Becton Dickinson, Franklin Lakes, NJ) and the needle was removed so only the tube remained. Bandage tape was attached to the port and adhered to the neck using super glue (Gorilla Glue, Cincinnati, OH). An interlinking 190-mm extension set (Baxter Healthcare, Deerfield, IL) was fastened to the port and secured to calves with 76-mm wide bandage tape (Elastikon, Johnson & Johnson, New Brunswick, NJ) loosely around the neck. The catheters were flushed with 3 mL of heparin saline solution containing 130 IU of heparin per mL of saline and capped immediately following placement and during the evening prior to the experiment.

2.3.3. Experimental Design

This experiment was performed as a generalized randomized complete block design. Fifty-four calves were used for this experiment. Nine calves from each of the 6 pens (i.e., blocks) were randomly assigned to one of three treatments: 1) local anesthetic lidocaine given as a cornual nerve block before cautery disbudding (AD; n = 18); 2) sham disbudding (SD; n = 18); or 3) oral herbal tincture (Dull It, Dr. Paul's Lab, Mazomanie, WI) administered before and after cautery disbudding (TD; n = 18). A local anesthetic was selected as a positive control treatment since this is the most widely used synthetic pain mitigation therapy used for disbudding calves on organic dairy farms, and the use of multimodal pain therapy is rarely implemented [26,68]. Treatments were balanced for sire breed and age (Table 2.1). The disbudding order within a pen was completely randomized.

Table 2.1. Distribution of calves by treatment and age, and treatment and sire breed

	Treatment [1]		
Item	AD	SD	TD
Day of age, mean ± SD	45 ± 6	44 ± 6	44 ± 6
Sire breed, count			
Holstein	6	8	8
Jersey	3	2	3
Montbéliarde	2	2	2
Normande	2	1	1
Swedish Red	5	5	4

[1] Treatments: AD = local anesthetic lidocaine 5 minutes prior to cautery disbudding; SD = sham disbudded; TD = oral tincture 2 minutes prior to and immediately after cautery disbudding.

The sample size for this experiment was determined using methods described by Guo et al. [189] and the GLIMMPSE software for repeated measures designs [190]. Only expected results for sham disbudding (i.e., SD treatment) and disbudding after a lidocaine cornual nerve block (i.e., AD treatment) were used to calculate sample size. The expected means and SD for key behaviors of head movements during disbudding and head shakes at 60, 120, 180 and 240 minutes after disbudding were from Graf and Senn [180]. The expected means and SD for cortisol at 60, 180 and 360 minutes after disbudding were from Stilwell et al. [182]. The expected effect sizes between treatments for head movements during disbudding, average head shakes after disbudding and average cortisol after disbudding were 1.1, 0.9 and 2.5, respectively. For the sample size calculations for head shakes and cortisol after disbudding, a linear exponent autoregressive model with a base correlation of 0.50 and decay rate of 0.30 was used in the GLIMMPSE online power and sample size software (https://glimmpse.samplesizeshop.org) to account for repeated measures. The estimated sample sizes needed to achieve a power greater than 0.80 for head movements during disbudding, head shakes after disbudding and cortisol after disbudding were 14, 6 and 8 calves per group, respectively. The maximum required sample size from these calculations was inflated by 30% to account for any potential dropped calves (14 × 1.30 = 18).

2.3.4. Treatment Administration

Ten minutes prior to disbudding, calves were restrained in a chute equipped with a head lock directly outside of the pen. Calves in the AD group received 5 mL of 2% lidocaine per side 5 minutes prior to disbudding. For each side, the needle (20-gauge × 19-mm) was inserted into the depression parallel to the temporal line pointed upward midway between the eye and horn bud, then 4 mL of lidocaine was administered into the cornual nerve and 1 mL was fanned around the nerve. Calves in the SD group did not receive any analgesic therapy, and disbudding was simulated by applying an unheated cautery iron (Inline Dehorner, Guilbert Express, New York, NY) to the horn buds of the restrained calf. Calves in the TD group received the tincture as directed by the manufacturer (2 mL of the herbal tincture sublingually 2 minutes prior to disbudding and 2 mL immediately after disbudding via a syringe). One person administered the lidocaine and tincture treatments throughout the experiment. Calves in a pen were cautery or sham disbudded 15 minutes apart and all calves in the experiment were cautery or sham disbudded between 10:00 and 14:40. Cautery disbudding was performed using a pistol grip cautery iron fueled by a butane/propane/propene mix (Express Dehorner, Guilbert Express, New York, NY). Cautery and sham disbudding were performed by one personnel who was blind to treatments for the cautery disbudded calves.

The dose and administration instructions for the tincture were in accordance with manufacturer guidelines. The tincture was previously marketed as a therapy to mitigate pain and stress related to castration and disbudding procedures for cattle, deer, goats, and sheep. It had been approved for use by many third-party organic certification agencies and had demonstrated popularity among organic dairy farmers for disbudding purposes. The tincture is comprised of (in order of greatest to least inclusion): ethanol, apple cider vinegar, white willow (*Salix alba* L.) bark, St. John's wort (*Hypericum perforatum* L.), chamomile (*Matricaria recutita* L.), arnica (*Arnica montana* L.) and fennel (*Foeniculum vulgare* Mill.).

2.3.5. Data Collection

Blood was collected at baseline (10 minutes prior to disbudding) and 1, 30, 90, 210 and 450 minutes following disbudding by discarding the first 3 mL and collecting the following 3 mL of blood, which was immediately transferred to serum separation tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and stored at 4 ºC. Tubes were centrifuged and serum was collected and maintained at −40 ºC until serology. Catheter patency was maintained by flushing with 3 mL of a heparin saline solution containing 13 IU of heparin per mL of saline after each blood collection.

Escape and struggle behaviors during disbudding were documented from audio/video recordings of calves from 5 pens (45 calves). A camera (iPad 3, Apple Inc., Cupertino, CA) was placed 1 m above calves to enable a full view of each calf's body during the disbudding procedure. Frequencies of tail wags, head movements, forces ahead, kicks, vocalizations, falls, and rears were counted for the duration of restraint from the moment the cautery iron contacted the first horn bud to the moment the cautery iron was released from the second horn bud. The duration of cauterization was also recorded.

Behaviors during and after disbudding were documented from video recordings of calves from 4 pens (36 calves). Two cameras were placed on opposite sides of each pen 1.5 m above the ground. For each calf, twenty-one 5-minute continuous observations were performed at baseline (60, 40 and 20 minutes prior to disbudding) and every 20 minutes following disbudding over the course of a 360-minute observation period. Frequencies of ear flicks, head jerks, head shakes, head rubs, oral behaviors, horn bud scratches and transitions, and durations of standing and ruminating were hand-recorded during each observation. An ethogram for behaviors recorded in the experiment are in Table 2.2. The ethological evaluation of disbudded calves was intended to assess pain, since behavioral adaptations can be observed in animals subjected to pain [191]. Tail wagging, head movements, forcing ahead, rapid leg movements and vocalizations are all behavioral adaptations frequently observed in ethological evaluations of calves during the cautery disbudding procedure [180,192], while ear flicking, exaggerated or rapid head movements, horn bud scratching, increased transitions between standing and lying, and variations in standing/lying, ruminating and oral manipulations are all behavioral adaptations commonly recorded in ethological evaluations of calves following cautery disbudding [56,182,193]. A single treatment-blinded observer assessed and documented behaviors. Interclass correlation coefficients of behavior observations for intra-reliability were > 0.90.

Table 2.2. Ethogram of behaviors assessed before, during and after the disbudding procedure

Behavior [1]	Description
Observations during disbudding	
Tail wag	A rapid lateral swing of the tail from one side of the body to the other
Head movement	A distinct movement of the head away from the cautery iron or upward. Not recorded during a rear or force ahead
Force ahead	A push forcefully forward
Kick	A lift and strike with a hind leg
Vocal	An oral sound, such a bellow or bawl
Fall	A complete drop to the ground or onto knees
Rear	An attempt to lift forelegs
Observations before and after disbudding	
Injury-directed	
Ear flick	A rapid movement of one or both ears. Not recorded during a head shake. Recorded as a new event once ears rested for > 2 seconds
Head jerk	An exaggerated head movement, such as a bob, jolt, or turn. Recorded as a new event once head rested for > 2 seconds
Head shake	A rapid head tilt from side-to-side while twisting neck. Recorded as a new event when head rested for > 2 seconds
Head rub	A back-and-forth movement of the head on any object. Not recorded during a horn bud scratch. Recorded as a new event when head rested for > 2 seconds
Horn bud scratch	A connection of the top of head with a hind hoof. Recorded as a new event when hoof returned to ground
Postural	
Standing	A stance where all hoofs are on the ground. Recorded as duration
Lying	A position where the body is in contact with the ground. Recorded as duration
Transition	A shift from standing to lying or lying to standing
Appetitive	
Oral manipulation	An interaction between an object and the mouth, such as grooming or manipulation of fixture. Not recorded during rumination. Recorded as a new event when object left mouth for > 2 seconds
Ruminating	A chewing jaw movement when calf was not feeding. Recorded as duration

[1] All behaviors are non-mutually exclusive and recorded as a frequency unless otherwise stated.

2.3.6. Cortisol Analysis

Blood serum samples were shipped over night in an insulated container with frozen carbon dioxide to the Veterinary Diagnostic Laboratory (Iowa State University, Ames, IA). Samples were analyzed for cortisol (CortiCote RIA Kit, MP Biomedical, Solon, OH) in duplicate and repeated if significant differences (i.e., inter-assay coefficient of variation > 18%) were present among duplicates. The coefficient of variation for the intra-assay variability was 17% and the coefficient of variation for the inter-assay variability was 13%. The limit of detection was 0.63 ng/mL.

2.3.7. Statistical Analyses

All data procedures and analyses were performed using version 4.0.2 of the RStudio software [194]. Pre-treatment baseline values were included as covariates for analyses of behaviors and cortisol evaluated after disbudding. Baselines for each behavior represented the average of the 3 observations performed prior to disbudding. Four missing cortisol and 43 missing behavior (ear flicks = 10, head jerks = 7, head shakes = 7, standing = 3, transitions = 3, ruminating = 6 and oral manipulations = 7) baseline values were imputed using the sample mean within pens, as described by White and Thompson [195]. Six (AD = 2, SD = 3 and TD = 1) and two (AD = 1 and TD = 1) calves were removed prior to the analyses of behaviors during and after disbudding, respectively, due to incomplete observations.

Separate models were evaluated for each outcome. All models included a covariate of *age*, a fixed effect of *treatment* and a random intercept for *pen*. Linear mixed models for the analyses of cortisol, cauterization duration and restraint duration were performed using the *lme* function of the *nlme* package [196]. Generalized linear mixed models analyzed behaviors using the *glmmTMB* function of the *glmmTMB* package [197]. For the analysis of cortisol after disbudding, the natural log transformation was applied as described by Osborne [198].

For the analyses of outcomes evaluated after disbudding, fixed effects also included the corresponding centered and scaled *baseline* value, *time*, and *treatment* × *time* interaction. Only 1 and 2 calves performed horn bud scratches and head rubs at baseline, respectively; therefore, the baseline covariate was removed for these analyses. To incorporate the dependency among observations within calf, the random intercept for *calf* was added. The heterogenous first order autoregressive covariance structure was used for the analysis of cortisol evaluated after disbudding to account for correlated repeated measures and heteroscedasticity among times. The first order autoregressive covariance structure was used for the analysis of behaviors evaluated after disbudding. Prior to the analyses of behaviors evaluated after disbudding, rarely observed outcomes of head shake, oral manipulation, standing, and rumination rates were aggregated into six 15-minute time intervals by taking the summation of 3 consecutive 5-minute time points. Similarly, horn bud scratch, head rub and transition rates were seldom observed and were therefore summed into a single 90-minute observation prior to analyses. Latency to lie down was recorded as the time lag corresponding to the first instance that lying was observed. Models for outcomes summed over all time points excluded fixed effects of *time*, *treatment* × *time* interaction, the random intercept for *calf* and the covariance structure. For the analyses of behaviors evaluated during disbudding, the log of the restraint duration was an offset variable. Vocalizations, falls, and rears were observed in only 10%, 2% and 2% of calves, respectively; and these outcomes are reported using descriptive statistics. Baseline cortisol and behaviors were analyzed separately.

For the analyses of behavior rates and latency to lie down, models were first evaluated with a Poisson distribution. Model fit was assessed by performing non-parametric overdispersion and zero-inflation tests from simulated null distributions using tools of the *DHARMa* package [199]; overdispersion or excess zeroes were deemed present when the corresponding observation to simulation ratio was > 1 ($p < 0.05$). If overdispersion was present, a negative binomial distribution with linear parameterization was used and the model was reassessed [200]. If excess zeros were present, a zero-inflated model with a single zero-inflation parameter applying to all observations was added. Poisson distributions were used for analyses of head movements and forces ahead during disbudding and ear flicks, head jerks, head rubs, head shakes, horn bud scratches and oral manipulations after disbudding. Negative binomial distributions were used for analyses of tail wags and kicks during disbudding, transition rates and latency to lie down after disbudding. The analyses of tail wags during disbudding and horn bud scratches after disbudding included a zero-inflation factor. Beta-binomial distributions were used for analyses of standing and rumination rates after disbudding.

Maximum likelihood estimates of the model parameters were used to determine least squares means. The F and Wald X^2 tests were used to test the significance of main effects for normally and non-normally distributed outcomes, respectively. The Tukey adjustment was applied to compare groups when the corresponding main effect had $p \leq 0.05$. For behavior outcomes, least squares means (LSM) and confidence intervals (CI) were transformed to the natural scale and incidence rate ratios (IRR) were used to compare groups.

2.4. Results

2.4.1. Behaviors During Disbudding

Cauterization and restraint durations were consistent among treatments (Table 2.3). Although personnel tried to achieve the same times for cauterization and restraint between treatments, the realized time the cautery iron was in contact with the horn buds (sum of right and left horn bud) was numerically greatest for calves in the SD group. The durations of cauterization and restraint were 5.9 seconds (SE = ± 0.7 seconds) and 10.8 seconds (SE = ± 1.3 seconds) when averaged across treatments, respectively.

Table 2.3. Means ± SE for effect of treatment on cauterization and restraint durations of calves undergoing disbudding procedures (*N* = 39)

	Treatment [1]			*F*-tests and *p*-values [2]	
Outcome, seconds	AD (n = 13)	SD (n = 12)	TD (n = 14)	Age (df_N = 1, df_D = 31)	Treatment (df_N = 2, df_D = 31)
Cauterization	5.6 ± 0.8	6.9 ± 0.9	5.2 ± 0.8	1.7 (0.20)	2.8 (0.07)
Restraint	11.6 ± 1.5	10.8 ± 1.5	9.9 ± 1.4	0.9 (0.35)	1.0 (0.37)

[1] Treatments: AD = local anesthetic lidocaine 5 minutes prior to cautery disbudding; SD = sham disbudded; TD = oral tincture 2 minutes prior to and immediately after cautery disbudding. [2] df_N = numerator degrees of freedom; df_D = denominator degrees of freedom.

Frequencies of behaviors recorded for the duration of disbudding restraint were similar among treatments (Table 2.4), indicating that restraint alone was a stressful event for calves and induced escape and struggle behaviors. Vocalization, fall, and rear behaviors were rarely observed. Vocalizations were not observed for calves in the AD but were observed in 7% and 23% of calves in the TD and SD groups, respectively. Falls were only observed for calves in the TD group (7%) and rears were only observed for calves in the AD group (7%).

Table 2.4. Means ± 95% CI for effect of treatment on behavior rates of calves during disbudding procedures (*N* = 39)

	Treatment [1]			X^2-tests and *p*-values
Behavior, events per 10-seconds [2]	AD (*n* = 13)	SD (*n* = 12)	TD (*n* = 14)	Treatment (df = 2)
Tail wags	12.5 [8.3, 18.9]	13.3 [8.9, 19.9]	13.6 [9.0, 20.5]	0.1 (0.95)
Head	2.9 [1.9, 3.9]	2.1 [1.3, 2.9]	1.9 [1.2, 2.7]	2.9 (0.23)
Forces ahead	0.3 [0.1, 0.8]	0.5 [0.2, 1.2]	0.5 [0.2, 1.1]	1.1 (0.56)
Kicks	0.5 [0.1, 1.5]	0.2 [0.0, 1.0]	0.3 [0.1, 1.1]	0.7 (0.69)

[1] Treatments: AD = local anesthetic lidocaine 5 minutes prior to cautery disbudding; SD = sham disbudded; TD = oral tincture 2 minutes prior to and immediately after cautery disbudding. [2] Behavior rates are reported as the number of events per 10 seconds of restraint. There was no effect of age on tail wags ($X^2_{(1)}$ = 0.2, p = 0.65), head movements ($X^2_{(1)}$ = 0.0, p = 0.97), forces ahead ($X^2_{(1)}$ = 0.0, p = 0.89) or kicks ($X^2_{(1)}$ = 0.6, p = 0.45).

2.4.2. Behaviors After Disbudding

Table 2.5 reports results for behaviors categorized into injury-directed, postural, and appetitive groups evaluated during the 360-minute observation period following disbudding.

Table 2.5. Means ± 95% CI for effect of treatment on behaviors of calves during the 360-minute observation period following disbudding procedures (*N* = 34)

Behavior	Treatment [1]			X^2-tests and *p*-values [4]		
	AD (*n* = 11)	SD (*n* = 12)	TD (*n* = 11)	Tr (df = 2)	Ti (df = 17)	Tr × Ti (df = 34)
Injury-directed						
Ear flicks, events per 5-minutes	-	-	-	4.9 (0.09)	30.7 (0.02)	72.7 (<0.01)
Head jerks, events per 5-minutes	2.1 [1.2, 3.5] [a]	0.9 [0.6, 1.5] [b]	1.4 [0.8, 2.4] [ab]	8.3 (0.02)	6.3 (0.99)	46.2 (0.08)
Head shakes, events per 15-minutes [2]	1.9 [1.1, 3.4] [a]	0.6 [0.4, 1.1] [b]	1.2 [0.7, 2.2] [ab]	7.7 (0.02)	2.9 (0.72)	10.3 (0.42)
Horn bud scratches, events per 90-minutes [3]	17.4 [5.9, 51.2] [a]	1.0 [0.2, 3.9] [c]	6.8 [2.2, 21.2] [b]	62.4 (<0.01)	-	-
Head rubs, events per 90-minutes [3]	1.8 [0.7, 4.6] [a]	0.6 [0.2, 1.8] [b]	2.1 [0.9, 5.2] [a]	11.5 (<0.01)	-	-
Postural						
Standing, seconds per 15-minutes [2]	84 [31, 205]	90 [36, 203]	62 [21, 172]	0.8 (0.67)	3.6 (0.61)	11.4 (0.33)
Transitions, events per 90-minutes [3]	4.5 [2.1, 6.9]	4.2 [2.0, 6.3]	5.3 [2.7, 8.0]	0.5 (0.78)	-	-
Latency to lie down, minutes	32 [25, 40] [a]	24 [19, 31] [ab]	20 [16, 26] [b]	8.0 (0.02)	-	-
Appetitive						
Ruminating, seconds per 15-minutes [2]	7 [1, 54]	36 [7, 165]	7 [1, 53]	2.8 (0.24)	3.0 (0.71)	13.7 (0.19)
Oral manipulations, events per 15-minutes [2]	0.4 [0.2, 0.9]	1.0 [0.5, 1.9]	0.3 [0.1, 0.8]	5.0 (0.08)	9.4 (0.09)	8.8 (0.55)

[a–c] Labeled means without a common letter within each row differ ($p \leq 0.05$). [1] Treatments: AD = local anesthetic lidocaine 5 minutes prior to cautery disbudding; SD = sham disbudded; TD = oral tincture 2 minutes prior to and immediately after cautery disbudding. [2] Observations were aggregated into 6 consecutive time intervals. X^2(Ti) df = 5; X^2(Tr × Ti) df = 10. [3] Observations were aggregated over observation period. [4] Tr = treatment; Ti = time; Tr × Ti = treatment × time interaction.

2.4.3. Injury-Directed Behaviors After Disbudding

Ear flicks, head jerks and head shakes were the most frequently observed injury-directed behaviors. In general, injury-directed behaviors were greatest for calves in the AD and lowest for calves in the SD group, while calves in the TD group had an intermediate response.

There was a significant treatment and time interaction for the analysis of ear flicks so means are reported in Figure 2.1. In general, the SD group had the lowest rate of ear flicks, while the AD and TD group had elevated ear flick rates following the disbudding procedure. There was an effect of baseline ear flicks ($X^2 = 6.3$, $p = 0.01$), such that calves that had greater ear flicks during the pre-treatment period also had greater ear flicks following the disbudding procedure. The AD group had 2.9 (95% CI = 1.0 to 8.3, $p = 0.04$), 5.1 (95% CI = 1.4 to 19.0, $p = 0.01$), and 6.9 (95% CI = 1.2 to 39.1, $p = 0.03$) times greater ear flick rates compared to the SD group at 180, 280 and 360 minutes after disbudding, respectively. The TD group had 3.9 (95% CI = 1.1 to 14.0, $p = 0.03$) and 5.5 (95% CI = 1.4 to 22.7, $p = 0.01$) times greater ear flick rates compared to the SD group at 140 and 340 minutes after disbudding, respectively. The TD and AD groups had similar ($p \geq 0.22$) ear flick rates at all time points except at 360 minutes after disbudding, where the AD group had 5.5 (95% CI = 1.4 to 22.6, $p = 0.01$) times the ear flick rate compared to the TD group.

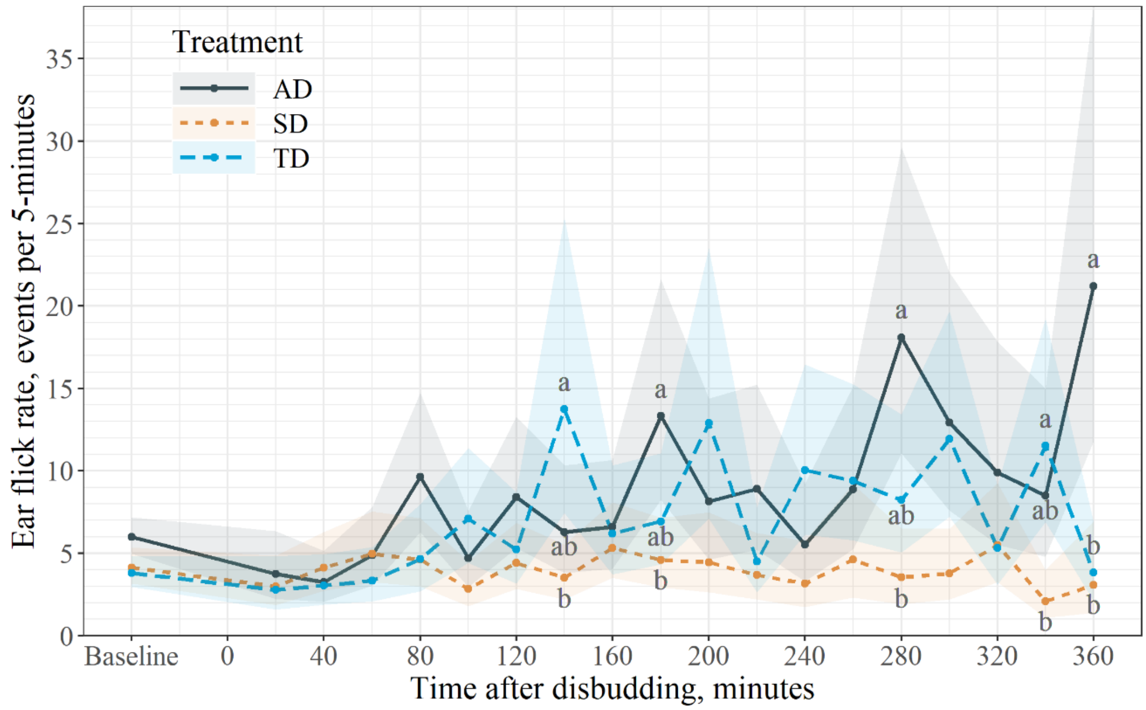

Figure 2.1. Means ± 80% CI for interaction of treatment and time on ear flick rates of calves during the 360-minute observation period following disbudding procedures (*N* = 34). Treatments: AD = local anesthetic lidocaine 5 minutes prior to cautery disbudding; SD = sham disbudded; TD = oral tincture 2 minutes prior to and immediately after cautery disbudding. Labeled means without a common letter within each time interval differ ($p \leq 0.05$).

The AD group had a 2.3 (95% CI = 1.1 to 4.8, $p = 0.03$) times greater head jerk rate than the SD group when averaged across all time points. The TD group had comparable ($p \geq 0.40$) head jerk rates to the other treatments throughout the experiment.

The AD group had a 3.0 (95% CI = 1.2 to 7.6, $p = 0.01$) times greater head shake rate than the SD group when averaged across all time points. The TD group had similar ($p \geq 0.24$) head shake rates to the other groups during the experiment.

Horn bud scratches and head rubs were the least observed injury-directed behaviors, yet calves in the AD and TD groups displayed greater ($p \leq 0.02$)

frequencies compared to calves in the SD group. The AD group had the greatest horn bud scratch rate compared to the other treatments, which was 17.7 (95% CI = 6.1 to 51.4, $p < 0.01$) times greater than the SD group and 2.5 (95% CI = 1.6 to 4.2, $p < 0.01$) times greater than the TD group. Furthermore, calves in the TD scratched their horn buds at a rate that was 7.0 (95% CI = 2.2 to 21.8, $p < 0.01$) times greater than calves in the SD group. There was an effect of age on horn bud scratch rate ($X^2 = 9.4$, $p < 0.01$), such that older calves were more likely to scratch their horn buds than younger calves. Head rub rates were similar ($p = 0.86$) for disbudded calves (AD and TD) regardless of treatment. The AD and TD groups had head rub rates that were 3.0 (95% CI = 1.2 to 7.8, $p = 0.02$) and 3.5 (95% CI = 1.4 to 8.7, $p < 0.01$) times greater than the SD group.

2.4.4. Postural and Appetitive Behaviors After Disbudding

Standing and transition rates were similar among treatments, but calves in the AD took 1.6 (95% CI = 1.0 to 2.4, $p = 0.03$) times longer to lie down after the disbudding procedure compared to calves in the TD group. Oral manipulation rates and rumination rates were similar among treatments.

2.4.5. Blood Serum Cortisol

Blood serum cortisol concentrations were greater ($p < 0.01$) for the TD group compared to the SD group at 10, 30 and 90 minutes after disbudding, and to the AD group at 30 minutes after disbudding (Figure 2.2). The effects of age, baseline cortisol and the treatment × time interaction had $p = 0.50$, $p < 0.01$, and

$p < 0.01$, respectively. There were no effects of age nor treatment for the analysis of baseline cortisol ($p \geq 0.36$). The TD group had 8.2 ng/mL (95% CI = −0.4 to 16.7 ng/mL, $p < 0.01$) greater cortisol compared to the SD group 10 minutes after disbudding, while the AD group had an intermediate outcome. The TD group had the greatest cortisol 30 minutes after disbudding, which was 20.1 ng/mL (95% CI = 8.1 to 31.1 ng/mL, $p < 0.01$) and 14.3 ng/mL (95% CI = 1.5 to 27.1 ng/mL, $p < 0.01$) greater than the SD and AD groups, respectively. The AD group also had 5.8 ng/mL (95% CI = −1.1 to 12.7 ng/mL, $p = 0.02$) greater cortisol compared to the SD group at 30 minutes after disbudding. The TD group had 4.5 ng/mL (95% CI = 0.4 to 8.6 ng/mL, $p < 0.01$) greater cortisol compared to the SD group 90 minutes after disbudding, while the AD group had an intermediate response. Furthermore, the TD and AD groups had similar ($p = 0.25$) cortisol values 90 minutes following disbudding.

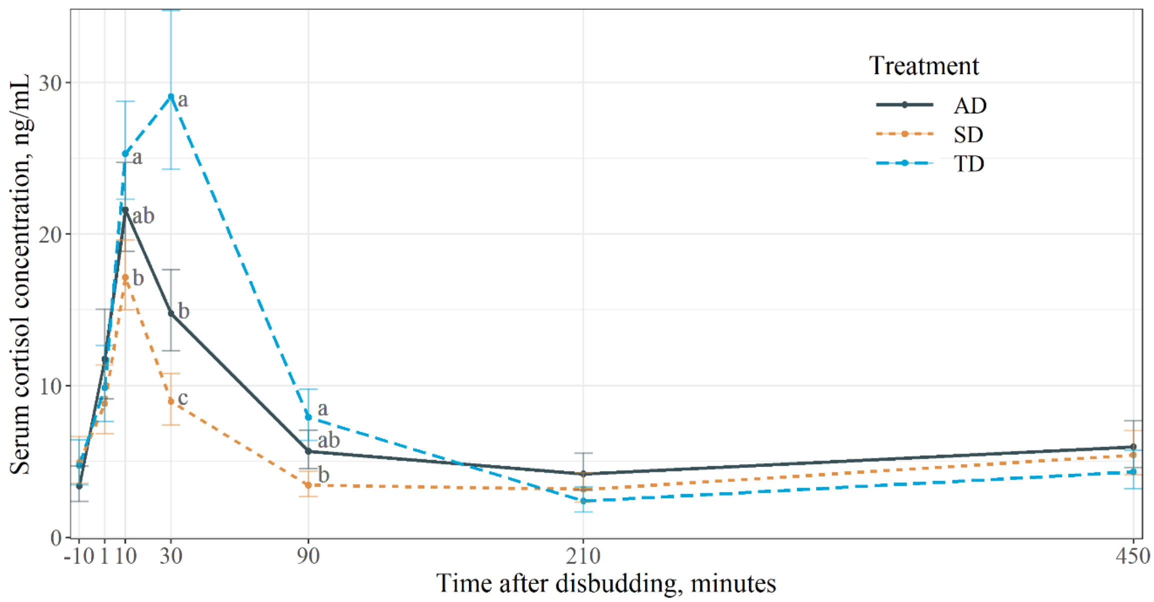

Figure 2.2. Means ± 80% CI for interaction of treatment and sampling time on blood serum cortisol concentration (*N* = 54). Treatments: AD = local anesthetic lidocaine 5 minutes prior to cautery disbudding; SD = sham disbudded; TD = oral tincture 2 minutes prior to and immediately after cautery disbudding. Labeled means without a common letter within each time point differ ($p \leq 0.05$).

2.5. Discussion

Contrary to our hypothesis, we observed no effect of treatment on behaviors evaluated during disbudding. The relatively short cauterization duration of approximately 6 seconds in this experiment may explain why behavioral differences were not apparent between calves that were sham disbudded and calves that were disbudded with lidocaine, but were in previous studies where the durations of cauterization were greater than 15 seconds [56,180]. Furthermore, the level of restraint required during the disbudding procedure may have suppressed behaviors in cautery disbudded calves. Intuitively, the handler performing the disbudding procedures was not blinded to cautery versus sham disbudding. Therefore, less restraint may have been used for sham-disbudded calves, resulting in the enhanced expression of behaviors and masking of behavioral differences between cautery and sham disbudded calves.

In general, calves disbudded with a local anesthesia had the greatest injury-directed behavioral response after disbudding, followed by calves disbudded with the tincture and sham disbudded calves. For the calves disbudded with a local anesthetic, head jerks and head shakes peaked at approximately 80 to 120 minutes after disbudding. This time period likely represents when sensitivity in the horn bud area returned, since the functional duration of lidocaine is approximately 90 minutes [185]. Huber et al. [184] also reported that a greater proportion of calves displayed head shakes and horn bud scratches during the 8-hour observation period following disbudding when they were administered with a local anesthetic prior to disbudding compared to sham disbudded calves.

Sham disbudded calves had a mean ear flick rate of 3.9 events per 5-minutes when averaged across all time points, which is greater than previous studies that report ear flick rates of ≤ 1.4 events per 5-minutes [79,182,184]. It was unclear whether these earlier studies were performed indoors where fly populations could have been suppressed. Since the current experiment took place outdoors during the summer, fly pressure and consequent avoidance behaviors may have exacerbated ear flick rates and masked differences between treatments [201]. Previous studies allude that ear flick behaviors may not be a completely reliable measure of pain following disbudding, such that inconsistent ear flick frequency outcomes are reported among varying levels of pain mitigation therapies [56,79,180,182,184].

Postural behavior rates of standing, lying and transitions were similar among treatments, but calves disbudded with the tincture were more likely to lie down compared to calves disbudded with a local anesthesia. Similarly, Faulkner and Weary [79] reported comparable lying rates among calves disbudded with varying levels of pain mitigation therapy over a 24-hour observation period, and Stilwell et al. [182] reported no effect of pain mitigation treatment on transitions between lying and standing postures. It is unclear why calves disbudded with the tincture were more likely to lie down sooner. Perhaps the first lying instance after disbudding may reflect pain in disbudded calves, but this phenomenon is currently not supported by research. The advertised calming effects of the tincture may have resulted in recumbency immediately following the procedure, which has been previously observed in disbudded calves that received a sedative [56,79]. However, plant constituents and their physiological effects have yet to be studied extensively in cattle. Potential sedation from the tincture may actually be problematic in terms of protecting animal welfare since pain-related behaviors could be concealed without actually providing any relief from pain [202,203].

Appetitive behavior rates were similar among treatments. Faulkner and Weary [79] also reported similar grooming, feeding and drinking rates among calves disbudded with varying levels of pain mitigation therapy. An early experiment reported that cautery disbudded calves that did not receive analgesia had decreased rumination rates during the 4-hour period following disbudding and increased rumination latencies compared to calves that were not disbudded [56]. Appetitive behavior differences among treatments were negligible in the current experiment and it remains unclear whether these findings were due to level of pain or another probable cause, such as lethargy that may have decreased behavioral responses.

Calves disbudded with the experimental tincture had the greatest cortisol response, followed by calves disbudded with the local anesthesia and sham disbudded calves. Calves that received the tincture peaked in cortisol at 30 minutes, whereas the calves disbudded with the local anesthesia and sham disbudded calves peaked at 10 minutes after disbudding. These results are similar to those reported by Graf and Senn [180], where cautery disbudding without analgesia resulted in a later cortisol peak compared to sham disbudding or cautery disbudding with a local anesthetic in calves. Some previous studies reported an elevated cortisol plateau for disbudded calves that received a local anesthesia [180–182], but this effect was not observed in the current experiment nor in another similar experiment [179]. It is possible that a secondary peak in cortisol occurred but was not apparent due to straggling sample intervals.

Observed behaviors did not reflect the high cortisol levels for cautery disbudded calves that received the experimental tincture, which may have multiple plausible explanations. It is possible that unexpected inactivity and recumbency observed in calves that received the tincture could be partially explained by stress-induced analgesia and learned helplessness [204]. Unusually low activity and inert behaviors have been previously documented in young animals following painful procedures, as indicated in evaluations of chemically disbudded calves [205,206], cautery disbudded calves [179] and castrated lambs [207].

The main possible plant-derived compound in the tincture includes a naturally occurring anti-inflammatory pro-drug (salicin) from willow tree (*S. alba*) bark [84], which is metabolized into salicylic acid in the body and has a similar anti-inflammatory mechanism to the nonsteroidal anti-inflammatories (NSAID) acetylsalicylic acid and sodium salicylate [208]. Given the small quantity of tincture administered, it is unlikely that salicin had any pain-reduction effect on calves. According to Coetzee et al. [80], a dose of 50 mg of oral acetylsalicylic acid per kg of body weight failed to attenuate peak cortisol concentrations after castration in 4- to 6-month-old cattle. Similarly, Mathurkar et al. [209] reported that an oral dose of 200 mg of sodium salicylate per kg of body weight failed to achieve a level of salicylic acid in the blood plasma necessary to have any analgesia effect in 6-month-old sheep (*Ovis aries* L.). Another possible compound in the tincture is found in St. John's wort (*H. perforatum*), which is commonly used as a replacement for standard anti-depressants to treat humans with mild to moderate depression [210]. The main constituents presumably responsible for the anti-depressant effects of St. John's wort are hypericin and hyperforin, yet their specific mechanisms of action are unclear and likely multifunctional [211]. Hypericin and hyperforin seem to inhibit the uptake of select neurotransmitters, such as gamma aminobutyric acid (GABA) and serotonin [212]. Inhibiting the uptake of GABA with gabapentin has successfully mitigated neuropathic pain in humans [213]. Likewise, inhibiting the uptake of serotonin may mitigate acute pain, as demonstrated in rodents given selective serotonin reuptake inhibitors [214,215]. Few studies have investigated the analgesic effects of neurotransmitter uptake inhibitors in disbudded or dehorned calves. The combined therapy of gabapentin and the NSAID meloxicam was previously evaluated for its potential in mitigating dehorning pain in calves. While analgesic effects of the combined therapy were not outstandingly superior to other therapies, authors of these studies suggested possible synergistic pharmacokinetic properties between meloxicam and gabapentin and solicited further investigation into this phenomenon [104,216,217].

Regardless of the potential constituents found in the experimental tincture, numerous studies agree that systemic anti-inflammatories or opioids alone are ineffective in reducing immediate acute surgical pain on young animals, as concluded under investigations with cautery disbudded calves [192], cautery disbudded goat (*Capra aegagrus hircus* L.) kids [218], castrated calves [219,220] and chemically disbudded calves [205,221,222]. Therefore, a local anesthetic should be administered to desensitize the horn bud area and effectively moderate pain during and immediately following disbudding [56,205].

Furthermore, when a local anesthetic is combined with a systemic NSAID, the immediate acute cortisol and injury-directed behavioral responses attenuate dramatically [79,181–184]. Authors of this experiment propose that organic producers may accomplish this multimodal therapy with lidocaine as a local anesthetic and flunixin meglumine as a NSAID [184], which are both approved for use in organic livestock according to regulations set forth by the USDA NOP. Perhaps the experimental oral tincture could provide multimodal pain relief when used in combination with other validated analgesic methods, such as lidocaine; however, further evidence is required to provide any indication of its utility.

2.6. Conclusions

Authors conclude that the restraint required for disbudding alone was a stressful event for calves, and neither the local anesthetic lidocaine nor the orally administered herbal tincture eliminated acute pain in disbudded calves as measured by observed behaviors and blood cortisol levels. Importantly, results also suggest that additional analgesic may be required to properly manage disbudding pain effectively. The experimental tincture examined in this experiment was evidently less effective than the local anesthetic in attenuating the cortisol response following disbudding, appeared to have no mechanism to mitigate pain during the disbudding procedure and may even suppress pain-specific behavioral responses for the hours following disbudding.

2.7. Publisher and Collaborator Recognition

A modified version of this chapter was accepted for publication in *Translational Animal Science* on 2021 March 7 [223]. I would like to express my gratitude to Bradley Heins for his contributions to this study and co-authorship for this publication. I would also like to express my gratitude to Dr. Paul Dettloff for supplying the experimental tincture for this project and to interns for their assistance in sample collection. This experiment was supported by Organic Agriculture Research and Extension Initiative (grant no. 2016-51300-25734/project accession no. 1010693) from the USDA National Institute of Food and Agriculture (Washington, DC).

Chapter 3: Effects of Oral White Willow (*Salix alba* L.) Bark and Intravenous Flunixin Meglumine On Prostaglandin E_2 in Healthy Dairy Calves

3.1. Summary

White willow bark (WWB) is commonly used in combination with other medicinal herbs and analgesics to alleviate inflammatory pain in disbudded calves under organic management, but there is no evidence on whether WWB has any effects on inflammatory biomarkers in calves. Therefore, the objective of this study was to determine if WWB affects the inflammatory biomarker prostaglandin E_2 (PGE2) in healthy dairy calves. A randomized crossover trial with 2 periods and 5 treatments was used for this experiment. A 7-day washout period was used to minimize carry-over effects. The treatments were: 1) 57.6 mg/kg oral WWB (LOW), 2) 115.1 mg/kg oral WWB (MED), 3) 230.3 mg/kg oral WWB (HIGH), 4) 2.2 mg/kg i.v. flunixin meglumine (FM) or 5) no treatment (NT). Calves (N = 25) were randomly assigned to receive one of the 25 treatment sequences. Blood samples were collected at 1, 2 and 4 hours after administration to determine prostaglandin E_2 (PGE2) and salicylic acid plasma concentrations. The WWB had 2,171 µg/g ± 4.3% relative standard error (0.22%) salicin. On average, calves in the FM (721 ± 274 pg/mL) treatment had lower PGE2 compared to calves in all other treatments. Calves in the NT (2,606 ± 271 pg/mL), LOW (2,509 ± 276 pg/mL), MED (2,343 ± 270 pg/mL) and HIGH (3,039 ± 270 pg/mL) treatments had similar PGE2 averaged across sampling times. Calves in the LOW (23.4 ± 1.9 ng/mL), MED (21.5 ± 1.9 ng/mL) and HIGH (23.3

± 1.9 ng/mL) treatments had similar maximum salicylic acid plasma concentrations. Results from this study indicate that the WWB doses used in this experiment were ineffective at achieving dose-dependent PGE2 and plasma salicylic acid plasma concentration responses.

Keywords: white willow bark, salicin, prostaglandin E_2, salicylic acid

3.2. Introduction

Dairy calves commonly experience painful disbudding procedures as a part of the standard of care. According to Urie et al. [224], approximately half (52%) of pre-weaned dairy calves in the US are disbudded, but only 28% of disbudded calves are given pain mitigation therapies for the procedure. Furthermore, a survey of 189 organic dairies in the US indicated that only 26% use a local analgesic, non-steroidal anti-inflammatory drug (NSAID) or sedation to relieve pain related to horn removal procedures [26]. Organic-approved options for pain management are limited to substances approved by the United States Department of Agriculture (USDA) National Organic Program (NOP), such as flunixin meglumine (§ 205.603). However, even those permitted by the NOP face barriers to common use, such as opposition by farmers, difficulty of administering and a lack of Food and Drug Administration (FDA) approval for use in cattle. Despite this reluctance to implement pain alleviation methods, some organic farmers have expressed interest in or currently implement plant-based alternatives [26,40].

An herbal tincture (Dull It, Dr. Paul's Lab, Mazomanie, WI) composed of ethanol, apple cider vinegar, white willow (*Salix alba* L.) bark, St. John's wort (*Hypericum perforatum* L.), chamomile (*Matricaria recutita* L.), arnica (*Arnica montana* L.) and fennel (*Foeniculum vulgare* Mill.) is currently used by many organic dairy producers as a therapy to mitigate disbudding pain and stress. However, the use of this tincture as a drug has not been approved by the FDA and is therefore is not approved for use. This herbal tincture was recently investigated as a therapy for modulating acute cautery disbudding pain in calves, in which results indicated that the herbal tincture did not reduce the cortisol response but reduced the behavioral response after disbudding compared to calves that received a lidocaine cornual nerve block [223]. To determine the possible mechanisms of this herbal tincture and other herbal therapies, single constituents of plants and their mechanisms should be investigated further.

Historically, white willow bark (WWB) has been used as an anti-inflammatory and analgesic, dating back to ancient civilizations [82]. Today, white willow bark is commonly used to treat painful conditions in humans [87–89]. As with all plants in the *Salix* genus, white willow bark contains salicylate compounds primarily comprised of salicin [83], which is converted to salicylic acid (SA) in the body when consumed orally [84]. Salicylic acid has inflammatory effects similar to synthetic salicylates, such as acetylsalicylic acid (i.e., aspirin) and sodium salicylate, in that it inhibits cyclooxygenases and prevents the formation of prostaglandins and inflammation [85,86]. However, there are currently no known peer-reviewed published studies indicating the usefulness of WWB for alleviating disbudding pain in calves.

Synthetic salicylates, such as aspirin and sodium salicylate, have historically been used as anti-inflammatories, antipyretics, and analgesics in cattle. Sodium salicylate administered i.v. at 50 mg/kg reduced cortisol concentrations when compared to untreated cattle following castration [80].

However, aspirin administered orally at 50 mg/kg did not attenuate cortisol [80]. Sodium salicylate dissolved in *ab libitum* drinking water at rates of 2.5 to 5.0 mg/mL 1 day prior to and 2 days after castration and dehorning was associated with improved ADG for 13 days and decreased cortisol concentrations for up to 6 hours following the procedures compared to calves that received no treatment [81]. Yet, despite the historical use of salicylates with cattle, they have never been formally approved by the FDA. Furthermore, unapproved products are currently marketed as if they are approved by the FDA and have undergone clinical research. In general, the leaves and bark of *Salix* spp. are considered safe for livestock consumption [90,91]. However, the effectiveness of WWB as a pain mitigation method in dairy calves is currently lacking scientific support. Therefore, the objectives of this study were: 1) to determine the salicin concentration of non-standardized products containing WWB that are currently or may be used for disbudding pain and 2) to determine the effects of i.v. flunixin meglumine and 3 oral doses of WWB on the inflammatory biomarker prostaglandin E_2 (PGE2) and salicylic acid plasma concentrations in healthy calves. The hypotheses of this study were: 1) that PGE2 would differ between calves given flunixin meglumine, no treatment, and low, medium, and high doses of WWB and 2) that maximum salicylic acid plasma concentration would differ between calves given low, medium, and high doses of WWB.

3.3. Materials and Methods

3.3.1. Salicin Concentrations

The salicin concentrations were determined in three products: 1) the aforementioned herbal tincture (Dull It, Dr. Paul's Lab, Mazomanie, WI), 2) an ethanol based WWB tincture (Mountain Rose Herbs, Eugene, OR) and 3) a dried WWB powder (Mountain Rose Herbs, Eugene, OR). Samples from each product were obtained from a single lot. Samples of the products were analyzed by a commercial laboratory (Eurofins EAG Materials Science, Maryland Heights, MO). Samples were prepared in duplicates and were analyzed by HPLC in duplicates; therefore, four replicates were analyzed per sample. For sample preparation, the tinctures were diluted in 50% aqueous methanol and passed through a 0.45-μm filter, while the powder was suspended in 50% aqueous methanol, sonicated for 10 minutes, centrifuged, and passed through a 0.45-μm filter. Samples were analyzed by HPLC equipped with a Zorbax SB-C18 phase column (5-μm particle size, 4.6-mm inner diameter. × 250-mm; Agilent, Santa Clara, CA) maintained at 35 °C. The injection volume was set to 5 μL and separation was performed at a flow rate of 1.0 mL/minute starting with a solvent composition of 5% acetonitrile, increasing linearly to 20% over 13 minutes. The solvent composition was increased to 80% acetonitrile over 3 minutes and held at 80% for 5 minutes before equilibrating to 5%. Salicin was detected at excitation and emission wavelengths of 210 and 268 nm, respectively. Salicin had a retention time of 8.43 minutes with a peak retention time of 0.1858% relative standard deviation (RSD) and peak area of 1.0041% RSD. For quantitation, a five-point calibration curve ranging between 10.44 and 100.13 μg/g was generated and had a R^2 greater than 0.99. The limit of detection was 10.44 μg/g. Quality control samples for the herbal tincture, white willow bark tincture and WWB powder had salicin recoveries 109, 101 and 93%, respectively. The average salicin concentration was greatest for the WWB powder (2171.2 μg/g ± 4.3% RSD) compared to the herbal tincture (17.6 μg/g ± 3.2% RSD) and the WWB tincture (143.3 μg/g ± 5.0% RSD). Therefore, the WWB powder was used for objective 2.

3.3.2. Animal Care

The experiment for objective 2 was conducted at the University of Minnesota West Central Research and Outreach Center in Morris, MN during December 2020 using 25 pre-weaned male calves. All procedures involving animals were approved by the University of Minnesota Institutional Animal Care and Use Committee (protocol number 2007-38250A). Calves were either a crossbreed composed of Viking Red, Montbéliarde and Holstein or a crossbreed composed of Jersey, Normande and Viking Red. Calves were (mean ± SD) 56 ± 15 d of age and weighed 85.7 ± 20.7 kg upon study initiation. Calves were housed in a single pen consisting of an indoor straw-bedded area (12.2 × 4.9 m) and an outdoor gravel area (10.7 × 4.9 m). Calves were fed pasteurized whole milk from an automated feeding system (CalfExpert Calf Feeder, Holm & Laue GmbH & Co KG, Westerronfeld, Germany). Calves had an 8-L daily allotment of milk in 2.4-L increments. Calves had *ad libitum* access to water and calf starter (18% CP).

3.3.3. Experimental Design

A randomized crossover trial with 2 periods and 5 treatments was used for this experiment. A 7-day washout period was used to minimize carry-over effects. The treatments were: 1) low WWB (LOW), 2) medium WWB (MED), 3) high WWB (HIGH), 4) flunixin meglumine (FM) or 5) no treatment (NT). The 25 calves (i.e., experimental units) were randomly assigned to receive one of the 25 treatment sequences (5 × 5 = 25). The WWB treatments were formulated based on the salicin concentration found in the WWB powder as previously described, such that the maximum number of boluses (size 12el, 7.5 mL capacity; Torpac, Fairfield, NJ) administered was 5. There are no known studies that use WWB in calves. Therefore, these doses were formulated based on what was presumed as feasible to give a calf because high doses that require numerous boluses may not be feasible for farmers based on limitations related to cost and labor. Authors agreed prior to the experiment that investigating doses that represent what farmers might give to their calves would be of most interest. Furthermore, this is the first experiment to investigate WWB in calves, and therefore, authors found it necessary to err on the side of precaution to avoid giving calves potentially large and unforeseen harmful doses. The FM treatment served as the positive control for this study since it is the only FDA- and organic-approved synthetic NSAID and it has known effects on PGE2 concentrations in calves [104]. Treatment sequences were balanced, and order of treatment was random. Calves were acclimated to handling 7 days prior to the study. On study days, treatment administration was staggered by 5 minutes. Calves in the LOW, MED and HIGH treatments received either 57.6, 115.1 or 230.3 mg/kg oral WWB powder in boluses corresponding to 0.125, 0.250 and 0.500 mg/kg salicin, respectively. Calves in the FM group received 2.2 mg/kg i.v. flunixin meglumine (Banamine, Merck Animal Health, Kenilworth, NJ). Calves in the NT group received no treatment. Handlers involved in collecting and processing blood samples from calves were treatment blinded.

3.3.4. Data Collection

Blood was collected immediately before and 1, 2 and 4 hours after treatment via jugular venipuncture (21-guage × 32-mm; Vacutainer Eclipse, BD Franklin Lakes, NJ). Collection times represented the periods of expected maximum SA serum concentration (1 and 2 hours) and half-life (4 hours) [97]. During each sampling, blood (4 mL) was collected in a sodium heparin tube (BD Franklin Lakes, NJ) for PGE2 determination and blood (4 mL) was collected in a K2 EDTA tube (BD Franklin Lakes, NJ) for SA determination. Tubes were gently inverted 8 to 10 times, immediately stored in a cooler on ice and processed within 30 minutes of collection.

For the PGE2 sample processing, whole blood (2 mL) was transferred from the collection tube to a 2-mL centrifuge tube (Fisher Scientific, Waltham, MA) containing 20 µL of 1 mg lipopolysaccharide (Sigma-Aldrich, St. Louis, MO) per 1 mL phosphate-buffered saline (Alfa Aesar, Haverhill, MA). The centrifuge tube was inverted 3 to 5 times and incubated for 24 hours in a 37 °C water bath (Isotemp GPD 05, Fisher Scientific, Waltham, MA). After incubation, tubes were centrifuged (HWLAB 1-12K mini multi speed desktop centrifuge, Fristaden Lab, Chicago, IL) at 400 × *g* for 10 minutes before plasma was transferred to cryovials (Fisher Scientific, Waltham, MA) and frozen at −80 ºC. For the SA sample processing, blood was centrifuged for 15 minutes at 2,500 rpm in a chilled centrifuge at 4 °C and plasma was transferred to cryovials and frozen at −80 ºC. Upon study completion, plasma samples were overnight shipped on dry ice to Analytical Chemistry Services (Iowa State University, Ames, IA) for analysis.

3.3.5. PGE2 Analysis

For PGE2 determination, a protein precipitation was performed on samples in preparation for competitive enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI). In short, plasma (93.75 µL) with 375 µL of high-performance liquid chromatography (HPLC)-grade methanol was centrifuged, and the supernatant was decanted into a 5-mL glass culture tube. The solvent was evaporated under a flow of nitrogen in a TurboVap LV (Biotage, Charlotte, NC) at room temperature. The dried extract was re-suspended in 375 µL of buffer to a dilution of 1:5. Samples were further diluted to 1:20 with buffer before analysis. Samples were analyzed in duplicate with an eight-point standard curve according to kit instructions. The assay had a detection range of 7.8 to 1,000 pg/mL. Samples were re-analyzed if the coefficient of variation was greater than 20% or if the value was not on the standard curve. Quality control samples were not run with this study, so intra-assay variability was not determined. The inter-assay variability was 10.0%. All curves were linear and had an average R^2 value of 0.99. Percent binding was 51% over all assays, and non-specific binding was 0.29%. The limit of detection was 7.8 pg/mL, and the limit of quantitation was 9.60 pg/mL.

3.3.6. Salicylic Acid Analysis

Salicylic acid concentration was determined using ultra HPLC (Thermo Vanquish Flex, Fisher Scientific, Waltham, MA) consisting of a binary pump, autosampler, column compartment, variable wavelength ultraviolet detector and a variable wavelength fluorescence detector. Plasma (0.2 mL) was aliquoted for extraction of calibrators, quality controls and samples. Calibrators were spiked into a blank matrix at 8 concentrations ranging from 20 to 5,000 ng/mL. Three quality controls were spiked into blank matrices at 150, 1,500 and 3,500 ng/mL. A volume of 20 µL of 12% formic acid was added to each extraction tube, followed by 2 mL of methyl tert-butyl ether. Tubes were placed on a multi-tube vortex mixer for 10 minutes followed by centrifugation for 5 minutes at 4 °C. The upper layer (1 mL) was transferred and concentrated to dryness at 25 °C. Samples were reconstituted in 0.1% aqueous formic acid. The mobile phases consisted of 1) 3.5 mM phosphate solution with 0.1% aqueous formic acid and 2) acetonitrile. Separation was accomplished using an aQ accucore column (2.6-µm particle size, 2.1 mm inner diameter × 100-mm; Fisher Scientific, Waltham, MA) maintained at 45 °C. The autosampler was maintained at 6 °C and the injection volume was set to 5 µL. The separation was performed at a flow rate of 0.3 mL/min at a starting solvent composition of 25% acetonitrile increasing linearly to 35% acetonitrile over 3.5 minutes. The solvent composition was then increased to 95% acetonitrile over 0.5 minutes and held at 95% acetonitrile for 2 minutes before equilibrating to 25% acetonitrile. Salicylic acid was detected at an excitation wavelength of 295 nm and an emission wavelength of 410 nm and had a retention time of 1.92 minutes (SD = 0.019 minutes). Thermo Chromeleon software (Fisher Scientific, Waltham, MA) was used to process quantitative results. A calibration consisting of 8 points between 20 and 5,000 ng/mL and a blank resulted in a linear curve with an R^2 of 0.99. The lower limit of quantification was 20 ng/mL. All quality control samples were calculated within 20% of their nominal value.

3.3.7. Statistical Analysis

All statistical analyses used the 1.4.1103 version of the RStudio software [194]. Analyses were performed using the *lmer* function of the *lme4* package [225]. For the analysis of PGE2, the model included fixed effects for *baseline PGE2* (continuous), *body weight* (continuous), *period* (2 levels), *time* (3 levels), *treatment* (5 levels) and the time and treatment interaction, and random intercepts for *calf* (25 levels) and *calf* within *period*. Baseline PGE2 was analyzed in a separate model with fixed effects of *body weight*, *period* and *treatment*, and a random intercept for *calf*. For the analysis of SA, the NT and FM treatments were removed, and the maximum SA value was identified for each calf. The model for maximum SA included fixed effects for period and treatment and a random intercept for calf. *Order of treatment* (continuous) and *breed* (2 levels) were considered as fixed effects candidates but were excluded from the models based on lack of improved model fit. Continuous predictors were centered and scaled to have a mean of 0 and SD of 1 for all models. The restricted maximum likelihood parameter estimates were used to calculate the least squares means (LSM) and standard error of the mean (SEM), and the *F*-tests were used to evaluate the significance of main effects. The Kenward-Roger approximation was used to calculate denominator degrees of freedom (df). The Tukey adjustment was applied to compare treatment means if the corresponding main effect had $p < 0.05$.

3.4. Results

Similar baseline PGE2 values (LSM ± SEM) were observed for calves in the FM (2,443 ± 442 pg/mL), NT (2,846 ± 444 pg/mL), LOW (3,170 ± 443 pg/mL), MED (2,825 ± 443 pg/mL) and HIGH (2,800 ± 441 pg/mL) treatments ($F_{4, 43} = 0.3$, $p = 0.85$).

For the analysis of post-treatment PGE2, there was no interaction between time and treatment ($F_{8, 90} = 2.0$, $p = 0.05$). However, there were effects of time ($F_{2, 90} = 3.8$, $p = 0.03$) and treatment ($F_{4, 37} = 11.5$, $p < 0.01$). When averaged across all post-treatment time points, calves in the FM group had lower PGE2 compared to all other treatments (Table 3.1). The PGE2 (LSM ± SEM) was greater (p = 0.03) at 2 hours (2,396 ± 164 pg/mL) compared to 4 hours (2,010 ± 164 pg/mL), while PGE2 at 1 hour (2,324 ± 164 pg/mL) had an intermediate value compared to the other time points ($p \geq 0.09$).

Table 3.1. LSM ± SEM for the effect of treatment on plasma concentrations of prostaglandin E_2 (PGE2) and maximum salicylic acid (SA) in calves (*N* = 25) enrolled in a randomized crossover trial with 2 periods and 7-day washout [1]

	Treatment [2]				
Plasma concentration	FM	NT	LOW	MED	HIGH
PGE2, pg/mL	721 ± 274 [b]	2,606 ± 271 [a]	2,509 ± 276 [a]	2,343 ± 270 [a]	3,039 ± 270 [a]
Maximum SA, ng/mL	-	-	23.4 ± 1.9	21.5 ± 1.9	23.3 ± 1.9

[a,b] Treatment means within a row are different at $p < 0.01$. [1] Blood samples for determining plasma concentrations of PGE2 and maximum SA were taken at 1, 2 and 4 hours after treatment administrations. [2] Treatments: FM = 2.2 mg/kg i.v. flunixin meglumine; NT = no treatment; LOW = 57.6 mg/kg oral white willow bark; MED = 115.1 mg/kg oral white willow bark; HIGH = 230.3 mg/kg oral white willow bark.

The LOW, MED and HIGH treatments had similar maximum SA (Table 1; $F_{2,7} = 1.2$, p = 0.36). Only 5 calves that received the WWB treatment achieved a SA plasma concentration greater than the lower limit of quantification (20 ng/mL), in which 4 received the HIGH treatment and 1 received the LOW treatment. Maximum SA concentrations were only observed at 1 hour (3 calves) and 2 hours (2 calves).

3.5. Discussion

This research is the first to report the use of WWB in calves. The WWB product used in this study had 2,171 μg/g (0.22%) salicin. But the concentration of salicin may vary between product lots. As expected, the FM treatment successfully reduced inflammatory mediators in calves, as indicated by lower PGE2 values compared to the NT treatment. However, none of the 3 doses of WWB reduced PGE2 and maximum SA plasma concentrations were similar among LOW, MED and HIGH treatments, indicating that the treatment doses were possibly too small. Furthermore, most calves who received the WWB treatments had undetectable SA plasma concentrations, indicating that the doses of WWB were too low to detect.

Salicin is the most notable medicinal compound in WWB extracts. After ingesting, salicin is converted to metabolites in the salicylate family, which can be detected in the plasma of blood. There are several compounds that are considered salicylates, but SA is the major metabolite that makes up total salicylates detected in the plasma after ingesting salicin. In a pharmacokinetics experiment of oral WWB in humans, salicylic acid was the major metabolite (86% of total salicylates) of salicin detected in the serum [97]. In Schmid et al. [97] humans with an average body weight of 69.4 kg consumed a total of 1,360 mg of standardized WWB extract (240 mg salicin) over 2 time points 3 hours apart. The maximum SA plasma concentration (8.4 µmol/L) was reached after the second dose at 4 hours, which was equal to 1.16 µg/mL given the molar mass of SA ($0.0084\ \mu mol/mL \times 138.121\ g/mol = 1.16\ \mu g/mL$).

There are very few studies on the pharmacokinetics and pharmacodynamics of salicin. However, similar compounds, such as aspirin and sodium salicylate, also form salicylate metabolites and have been studied more intensively. The minimum total salicylate plasma concentration needed for analgesia in calves was previously estimated to be 25 to 30 μg/mL [80,98]. Since SA makes up an estimated 86% of total salicylates in the plasma after consuming salicin [97], the estimated minimum SA plasma concentration needed for analgesia in calves is approximately 21.5 to 25.8 μg/mL.

Previous studies of aspirin and sodium salicylate administered orally in ruminants suggest that greater doses than those of the present experiment are needed, coupled with more frequent administration. For example, single doses of aspirin in calves (50 mg/kg) and sodium salicylate in sheep (200 mg/kg) both failed to achieve plasma salicylate concentrations above 10 μg/mL [80,209], but aspirin at 100 mg/kg every 12 hours maintained plasma salicylate concentrations greater than 30 μg/mL in dairy cows [98]. Similarly, two daily aspirin doses of 200 mg/kg over the first 2 DIM reduced clinical metritis at 7 and 14 DIM [226], and 3 daily sodium salicylate doses of 185 mg/kg over the first 3 DIM increased early-lactation milk yield [227].

The area under the curve of SA plasma concentration obtained in Schmid et al. [97] after humans consumed WWB extract corresponding to 240 mg salicin (13.67 μg × h/mL) was similar to that expected after a single aspirin dose of 80 mg (12.60 μg × h/mL) and 100 mg (14.6 μg × h/mL) in humans [228,229]. Therefore, the estimated dose of salicin can be estimated by multiplying the aspirin dose by a factor of 2.6 to 2.8. Furthermore, aspirin doses of 100, 300 and

500 mg in humans had a linearly proportional relationship with the area under the curve and maximum concentration for plasma SA [229]. Mathurkar et al. [209] compared 2 oral doses of sodium salicylate in sheep and reported that 100 and 200 mg/kg yielded maximum SA plasma concentration values of 4.22 and 8.27 μg/mL, respectively. Based on the previous information and a linearly proportional relationship between dose and maximum concentration, calves would need sodium salicylate at a dose of approximately 520 mg/kg to reach the minimum SA plasma concentration needed for analgesia in calves (21.5 μg/mL). Alternatively, a total aspirin dose of 400 mg/kg given over a the course of several time points may also be adequate at reducing inflammatory biomarkers [226]. After multiplying these doses by a factor of 2.6 to 2.8, the estimated dose range of salicin needed for analgesia in calves is 1,040 to 1,456 mg/kg. The decided dose could be given over several time points to prevent gastrointestinal upset and stress to the calves. Previous studies use maximum single aspirin and sodium salicylate doses of 200 mg/kg [209,226], so salicin doses greater than 200 mg/kg at a single time point should be administered with precaution.

The estimated amount of salicin needed to achieve analgesia in calves is quite large considering that WWB has a minute amount of salicin. Even if a standardized WWB extract, such as a 15% salicin product was used, it would have to be given at a total dose of approximately 6,933 to 9,707 mg/kg (equivalent to 1,040 to 1,456 mg/kg of salicin). This dose could potentially be given over 1 to 3 days in drinking water or milk as demonstrated with aspirin and sodium salicylate in other studies [226,227]. However, this method may be impracticable considering time and financial constraints. Furthermore, there is currently no evidentiary support on whether WWB at high doses given over several days has any effect on inflammatory biomarkers in calves. Furthermore, other constituents of WWB might be toxic and have unknown pharmacokinetics and therefore withdrawal times. In fact, it is possible that sustained high doses of WWB may have negative effects on health and welfare, such as gastrointestinal upset and consequent increased inflammation, as demonstrated in adult cattle given aspirin orally [230].

3.6. Conclusions

In conclusion, the results of the current experiment reveal that products containing non-standardized WWB have a very small amount of salicin, and the necessary dose of WWB to reduce inflammatory biomarkers and achieve a SA plasma concentration required for analgesia in calves was not determined. In fact, the WWB doses evaluated in the present experiment were likely far smaller than what is required for an appropriate dose-dependent response. The proper WWB dose for analgesia in calves is untested and may possibly have unforeseen negative effects on animal wellbeing. Further research should focus on finding a dose of WWB or salicin that achieves a SA plasma concentration necessary for analgesia in calves before testing the efficacy of WWB under farm settings.

3.7. Publisher and Collaborator Recognition

A modified version of this chapter was submitted for publication in *JDS Communications* on 2021 June 10. I would like to express my gratitude to Bradley Heins, Kirsten Sharpe, and Marcia Endres for their contributions to this study and co-authorships for this publication. I would also like to thank Dr. Mateus Peiter, Brandi Gednalske, Teralyn Johnson and Morgan Mathews for their assistance with sample collections, Madison Bacon for her contribution to the manuscript and the West Central Research and Outreach Center staff for providing animal care. This work is supported by Organic Transitions (ORG) [grant no. 2020-51106-32357/project accession no. 1023662] from the USDA National Institute of Food and Agriculture.

Chapter 4: Effects of Pre-Parturient Iodine Teat Dip Applications on Modulating Aversive Behaviors and Mastitis in Primiparous Cows

4.1. Summary

Heifers and their human handlers are at risk for decreased welfare during the early lactation period. This experiment investigated pre-parturient teat dipping and parlor acclimation to reduce mastitis and aversive behaviors in early lactation heifers. Three weeks prior to calving, heifers were randomly assigned to receive either: 1) a weekly 1.0% iodine-based teat dip in the parlor (trained; $n = 37$) or 2) no treatment (control; $n = 30$). For the first 3 days of lactation, heifers were milked twice daily, and treatment-blinded handlers assessed behaviors and clinical mastitis. Aseptic quarter milk samples were collected within 36 hours of calving and analyzed for pathogens. Control heifers had (OR ± SE) 2.2 ± 0.6 times greater ($p < 0.01$) odds of kicking during milking. Trained heifers had (OR ± SE) 1.7 ± 0.4 times greater ($p = 0.02$) odds of being very calm during milking, while control heifers had 2.2 ± 0.8 and 3.8 ± 2.1 times greater ($p < 0.04$) odds of being restless and very restless or hostile during milking, respectively. Quarters of control heifers had (OR ± SE) 5.4 ± 3.4 greater ($p < 0.01$) odds of intramammary *Staphylococcus aureus* infection, yet clinical mastitis was similar among treatments. The results indicate that teat dipping in the parlor weekly for 3 weeks before calving may alleviate some aversive milking behaviors and protect against early lactation *S. aureus* intramammary infections.

Keywords: human–animal relationship, training, acclimating, heifer, primiparous, dairy, behavior, welfare, udder health, mastitis

4.2. Introduction

The periparturient period is particularly challenging for heifers. For one, the new experience of being milked may be distressing for some heifers. This may not only jeopardize animal welfare, but also endanger the safety of human handlers, as distressed heifers may kick off milking clusters, kick at handlers and display other aversive behaviors that interfere with milking efficiency. This increases the chance of injury to handlers and the risk of mastitis for the animal [106,108–110]. In addition, heifers are susceptible to intramammary infections (IMI) and clinical mastitis during this time [112–114]. While late gestation IMI and early lactation mastitis are associated with an economic loss due to treatment costs [115], production loss [231,232], risk of future infections [117,118], reduced reproductive performance [116] and increased risk of culling [136,233], negative affective states and poor milking behavior may also represent an economic loss due to risk of IMI [108], decreased milk productivity [119] and the risk of early culling [109]. Therefore, preventing early lactation distress and mastitis in heifers may be of interest in terms of improving animal welfare and economic benefit.

Several strategies have been studied to reduce the behavioral reactivity of heifers and improve the human-animal relationship. These include the positive handling of heifers and familiarizing them with the milking parlor before calving [109,120–125]. For example, the positive handling of heifers during the calving process reduced the number of flinch, step and kick responses performed during milking procedures over the first 20 weeks of lactation [109]. Bertenshaw et al.

[121] found that spending 30 to 245 minutes brushing heifers during the last 6 weeks of gestation can reduce kicking behaviors during milking procedures up to 4 weeks after parturition. Kutzer et al. [123] showed that pre-parturient training, which consisted of the introduction to the milking herd at least 10 days before calving and at least 10 visits to the parlor where milking staff provided tactile contact to the udder on the milking platform, reduced post-parturient aversive behaviors in heifers. However, intensive habituation protocols such as the ones described in previous experiments may not be feasible for many farms due to labor and infrastructure restraints, so it is important to devise a simpler habituation protocol that fits within the capabilities of dairy farms.

Protocols to prevent IMI and clinical mastitis have been examined using a variety of pre-parturient techniques, such as internal teat sealants [113,126–128], antibiotic therapies [129–133], milking [125,134,135] and repeated applications of teat dip or spray [136–138]. However, many of these methods, including internal teat sealants and antibiotics, are not allowed for use in organic dairy animals in the United States. Furthermore, labor limitations prevent the implementation of intensive protocols on most farms, especially those that house heifers on pasture. Therefore, the current protocol for preventing udder health issues in pre-parturient heifers needs modification to be feasible.

The objectives of this study were to determine whether weekly pre-parturient teat dipping in the milking parlor would modulate behavioral responses and decrease the mastitis and IMI risk of heifers over the first 3 days of the lactation period. The hypothesis of this experiment was that heifers that received weekly pre-parturient teat dipping in the milking parlor would have different behaviors and udder health during the first 3 days in milk (DIM) compared to heifers that received no pre-parturient treatment.

4.3. Materials and Methods

4.3.1. Animals and Housing

The University of Minnesota Institutional Animal Care and Use Committee approved all animal care and procedures specific to this experiment (protocol number 1906-37134A).

The experiment was conducted from March to November 2018 at the University of Minnesota West Central Research and Outreach Center (Morris, MN) using heifers in conventional and organic dairy research herds. Details on calf housing and care are described in Kienitz et al. [188]. Heifers used in this study were either pure Holstein or one of two crossbreeds, as described by Heins et al. [187].

Heifers were housed on pasture from 6 months of age and were supplemented with a total mixed ration during the nongrazing season. Heifers were first artificially inseminated after reaching 14 months of age during the winter (December to February) and summer (June to August) breeding seasons for the subsequent fall and spring calving seasons, respectively. Heifers were culled from the herd if they did not become pregnant after 2 breeding seasons.

Pregnant heifers were moved to a maternity pen 4 weeks prior to their calving due date. The range for age at calving was 691 to 791 days (mean ± SD = 726 ± 20 days). Heifers were housed in a compost-bedded pack barn and an outdoor straw pack for 24 hours after calving for health monitoring. Details on theses housing systems are described by Sjostrom et al. [234]. Twenty-four hours after calving, heifers moved into their respective conventional or organic lactating herd. The conventional or organic status of the heifer was based on the status of the heifer's dam.

Details on the conventional and organic lactating herd housing and feeding management are described by Minegishi et al. [235]. In brief, the organic herd was housed on pasture from May to October, where they had ad libitum access to forages for grazing, water and minerals and were fed 2.72 kg per head of organic corn daily. From November to April, the organic herd was housed in a compost-bedded pack barn or outdoor straw pack, where they were fed an organic total mixed ration. Meanwhile, the conventional herd was housed in an uncovered dry-lot from May to October and in a compost-bedded pack barn or outdoor straw pack from November to April. The conventional herd was fed a conventional total mixed ration.

Heifers were milked twice daily at 06:00 and 17:00 in a swing-nine parabone milking parlor after calving. At each milking, heifers were pre- and post-dipped with a 1.0% iodine-based teat dip. Teats were not dried before heifers exited the milking parlor. If the ambient outdoor temperature was less than −10

°C, heifers were post-dipped with an organically approved restricted-use chlorhexidine powder teat dip to prevent frostbite.

4.3.2. Experimental Design

This study used a generalized randomized complete block design where the season (spring and fall) served as the blocking factor. Before calving, heifers were allocated randomly to one of two treatment groups: 1) trained (*n* = 37) or 2) control (*n* = 30). Treatments were balanced for calving date. The number of animals per treatment according to breed, season and herd is listed in Table 4.1. Heifers allocated to the training treatment received a training program in the swing-nine milking parlor once weekly for 3 consecutive weeks until calving, whereas those in the control treatment remained in their home pen.

Table 4.1. Distribution of animals by treatment and group

	Treatment	
Group	Trained (*n* = 37)	Control (*n* = 30)
Breed [1]		
Holstein	11	9
MVH	16	18
NJV	10	3
Season		
Fall	25	21
Spring	12	9
Herd		
Conventional	12	21
Organic	25	9

[1] MVH = Montbéliarde, Viking Red and Holstein crossbred; NJV = Normande, Jersey and Viking Red crossbred.

Training began 4 weeks prior to the expected calving date of heifers. On each Tuesday between 12:00 and 14:00, heifers to be trained were brought to the parlor in groups of 6 to 9, wherein they could investigate the holding pen for 15 minutes. Then, they were loaded into the milking parlor by a handler using gentle stockmanship practices, which included quiet voice prompts, gentle hand and arm movements and light tactile force with hands if necessary. The parlor holding gate was then brought down to secure heifers in place to simulate normal milking. Then, each teat was cleaned with a single use towel and dipped with a 1.0% iodine-based barrier teat dip (Chem-Star Io-Soft 1000 + 10, Ecolab, St. Paul, MN) by a handler. Heifers remained in the parlor for 5 minutes until they were released to return to their home pen. Trained heifers received either 3 or 4 training sessions based on whether they calved near or before their expected calving date.

4.3.3. Data Collection

After calving, heifers were observed during each milking for 3 days. Treatment-blinded farm staff scored heifers according to similar methods described by Tulloh [236] for parlor entry ease (1 = willing; 2 = willing but hesitates; 3 = requires crowd gate prompt; 4 = requires handler to enter holding pen) and milking ease (1 = very calm; 2 = calm; 3 = restless; 4 = very restless; 5 = hostile). While heifers were in the parlor, farm staff recorded whether the following behaviors were performed: stomp, kick, defecate and kick off milker. An ethogram for these behaviors is provided in Table 4.2. Farm staff also assessed heifers for udder edema and clinical mastitis at each milking. Udder edema was defined as a swollen and firmer udder base without abnormal milk. Clinical mastitis was scored by visual observation of stripped milk from each quarter as follows [237]: normal (score 0) = milk is normal; mild (score 1) = milk contained flakes, clots or serum; moderate (score 2) = mild mastitis accompanied by swelling or redness of the mammary gland or quarter; or severe (score 3) = moderate mastitis accompanied by systemic signs of illness such as depression, anorexia, dehydration and/or fever. The 6 farm staff workers who observed and recorded data had at least 90% agreement on all recorded outcomes with the principal investigator of the study, which was assessed at the beginning of the fall and spring calving seasons.

Table 4.2. Ethogram of behaviors recorded during milking. Behavior descriptions were adapted from Eicher et al. [125].

Behavior	Description
Stomp	A raising and lowering of the foot in one place
Kick	A forward or sideways leg movement, without dislodging claws
Defecate	A discharge of feces
Kick off milker	A kick that causes the claws to fully or partially dislodge

After colostrum was collected, aseptic quarter milk samples were collected within 36 hours of calving and analyzed for pathogens at the University of Minnesota Veterinary Diagnostic Laboratory (St. Paul, MN). Before collection, loose manure, dirt, and bedding particles were brushed off from the udder and teats, and teats were dipped with a 1.0% iodine pre-dip. Thirty seconds after the dip was applied, teats were wiped clean with a single-use paper towel and the first 3 to 4 streams of milk from each teat were discarded. The apex of each teat was repeatedly scrubbed with a new gauze pad soaked in 70% isopropyl alcohol until a withdrawn pad was unsoiled. Milk (20 to 30 mL) was collected in sterile plastic tubes. During the sampling process, disposable gloves were changed between heifers. Samples were frozen within 24 hours of collection and were later shipped overnight with frozen gel packs to the lab for analysis within a month of collection.

For each quarter sample, a volume of 0.1 mL of milk was swabbed onto each section of a half-plate containing either Factor media (Gram-positive selective; University of Minnesota, St. Paul, MN) or MacConkey II (Gram-negative selective; Becton Dickinson, Franklin Lakes, NJ). The plates were incubated at 37 °C for 48 hours. After 24 hours, the plates were examined for growth and identification procedures were performed. After 48 hours, the plates were re-examined for growth and identification if new growth was present. For each sample, the level of bacteria growth was assessed and categorized as either no (< 1 colony forming units (CFU)/0.1 mL), low (1 to 10 CFU/0.1 mL), medium (11 to 50 CFU/0.1 mL) or high growth (> 50 CFU/0.1 mL). A sample was considered contaminated if more than 2 types of bacteria were present. Contaminated samples were not evaluated for bacteria species. For plates with growth, the colonies were differentiated by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI Biotyper, Bruker, Billerica, MA) [238]. Identification was performed to the species level when the CI was at least 2.00, and to the genus level if the CI was 1.80 to 1.99.

Pathogens were categorized according to Passchyn [147]. *Staphylococcus chromogenes*, *Staphylococcus sciuri*, *Staphylococcus hominis*, and other non-*aureus* (and coagulase negative) *Staphylococcus* spp. were combined into a single category of coagulase-negative staphylococci (CNS) [239]. The major contagious pathogen was *Staphylococcus aureus*. The other major pathogens were *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Enterobacter cloacae* and *Escherichia coli*. Coagulase-negative staphylococci, non-*agalactiae Streptococcus* spp., *Bacillus* spp., *Enterococcus faecalis*, *Serratia* spp. and unidentified Gram-positive cocci were the minor pathogens. Twenty-nine quarter samples had 2 species of bacteria identified, so only 1 species was kept for subsequent analyses, as described by Parker et al. [113]. When there was a mixed major and minor pathogen infection present (8 quarters), the quarter was defined as being infected with the major pathogen. In 1 quarter, there were 2 major pathogens isolated (*S. aureus* and *S. uberis*), so *S. aureus* was the species used in further analysis. When there were 2 minor pathogens present at differing levels (11 quarters), the quarter was defined as being infected with the pathogen of greater CFU in subsequent analyses. In 9 quarters, two minor pathogens at the same level were isolated (CNS and *Bacillus* spp. in 7 quarters; CNS and Gram-positive cocci in 1 quarter; and *Serratia* spp. and *Bacillus* spp. in 1 quarter), so CNS or *Serratia* spp. were the species used in further analysis.

4.3.4. Data Analysis

Data collected during parlor visits were aggregated across milkings. Similarly, udder health data from milk samples were aggregated across quarters. Some of the outcome variables were coerced into categorizes for the analysis. Entry ease scores 3 and 4 (score > 2) and milking ease scores 4 and 5 (score > 3) were combined into single score categories. Clinical mastitis and udder edema data were dichotomized into either absent during all milkings or present during at least 1 milking over the 3-day observation period. *S. dysgalactiae*, *S. uberis*, other non-*agalactiae Streptococcus* spp., *Bacillus* spp., *E. cloacae*, *E. faecalis*, *E. coli*, *Serratia* spp. and unidentified Gram-positive cocci were categorized as other environmental pathogens. Heifers with all quarter samples contaminated (10 heifers) were removed from the quarter milk analyses. Furthermore, seven and 63 quarter samples were excluded from the analysis due to being either missing or contaminated, respectively [240]. Therefore, 159 quarters were evaluated.

Analyses were performed in RStudio (version 1.3.1103) [194] with logistic regression using the *glm* function. The fixed effects were *age at calving* (continuous), *season* (2 levels), *breed* (3 levels), *herd* (2 levels) and *treatment* (2 levels). For the analyses of behaviors, the fixed effect of *clinical mastitis* (2 levels) was included in the models. Likelihood ratio tests (LRT) were used to assess the significance of effects by comparing full and reduced models [241]. Significance was declared when $p < 0.05$. Marginal means and CI for behaviors are reported as probability values back-transformed from the logit scale. Odds ratios (OR) and SE are used to compare treatment groups.

4.4. Results

4.4.1. Behaviors

Treatments were different for kicks and milking ease, while stomping, defecating, kicking the milking unit off, and entry ease were similar between treatments (Table 4.3). In general, trained heifers had preferred behaviors even after accounting for clinical mastitis. Control heifers had (OR ± SE) 2.2 ± 0.6 times greater odds of kicking during milking procedures compared to trained heifers. Trained heifers had (OR ± SE) 1.7 ± 0.4 times greater odds of being very calm during milking (milking ease score = 1) compared to control heifers, while control heifers had 2.2 ± 0.8 times greater odds of being restless during milking (milking ease score = 3) and 3.8 ± 2.1 times greater odds of being very restless or hostile during milking (milking ease score > 3) compared to trained heifers. There was an effect of calving age on stomping ($X^2_{(1)} = 5.3$, $p = 0.02$), kicking ($X^2_{(1)} = 2.2$, $p = 0.03$) and kicking off the milking unit ($X^2_{(1)} = 8.2$, $p < 0.01$), in which older heifers were more likely to perform this behavior.

Table 4.3. The probabilities (95% CI) of behaviors occurring during milkings and of the development of clinical mastitis and udder edema over the first 3 days in milk in heifers receiving a weekly 1.0% iodine-based teat dip in the parlor 3 weeks prior to calving (trained) or no treatment (control) based on logistic regression

	Treatment		**Treatment effect** [1]		**Odds ratio (OR) ± SE**
Behaviors and udder health, % probability	Train (n = 37)	Control (n = 30)	$X^2_{(1)}$	p-value	Train *vs* control
Stomp	62.0 [54.1, 69.3]	65.4 [56.6, 73.2]	0.4	0.53	0.9 ± 0.2
Kick	16.5 [11.4, 23.2]	29.8 [22.1, 38.9]	7.0	<0.01	0.5 ± 0.1
Defecate	9.6 [5.9, 15.3]	12.0 [7.3, 19.1]	0.5	0.49	0.8 ± 0.3
Kick off milker	4.0 [1.8, 8.6]	5.7 [2.8, 11.6]	0.5	0.47	0.7 ± 0.4
Entry ease [2]					
1	68.0 [59.3, 75.6]	61.7 [52.4, 70.3]	1.2	0.27	1.3 ± 0.3
2	14.1 [9.3, 20.6]	21.9 [15.2, 30.4]	2.9	0.09	0.6 ± 0.2
> 2	13.6 [8.3, 21.4]	13.3 [8.1, 21.0]	0.0	0.93	1.0 ± 0.3
Milking ease [3]					
1	53.0 [45.1, 60.8]	40.2 [31.9, 49.1]	5.1	0.02	1.7 ± 0.4
2	35.0 [28.0, 42.8]	34.5 [26.6, 43.3]	0.0	0.92	1.0 ± 0.2
3	6.9 [3.7, 12.3]	13.8 [8.4, 21.8]	4.4	0.04	0.5 ± 0.2
> 3	2.4 [0.9, 6.3]	8.3 [4.5, 15.0]	6.0	0.01	0.3 ± 0.2
Clinical mastitis	35.3 [19.4, 55.2]	23.7 [10.1, 46.3]	0.8	0.37	1.8 ± 1.1
Udder edema	6.3 [1.6, 22.1]	17.0 [5.9, 40.2]	1.8	0.18	0.3 ± 0.3

[1] Chi-square statistic of likelihood ratio test (LRT). [2] 1 = willing; 2 = willing but hesitates; > 2 = requires crowd gate prompt or requires handler to enter holding pen. [3] 1 = very calm; 2 = calm; 3 = restless; > 3 = very restless or hostile.

Clinical mastitis influenced some behaviors, in which heifers with clinical mastitis were more likely to hesitate while entering the parlor, kick during milking, and be very restless or hostile during milking compared to heifers without clinical mastitis. Heifers with clinical mastitis had (OR ± SE) 2.3 ± 0.7 times greater odds of kicking ($X^2_{(1)}$ = 7.7, p < 0.01), 3.0 ± 1.0 times greater odds of hesitantly entering the parlor (entry ease score = 2) ($X^2_{(1)}$ = 11.6, p < 0.01) and 2.7 ± 1.4 times greater odds of being very restless or hostile during milking (milking ease score > 3) ($X^2_{(1)}$ = 3.9, p = 0.048) compared to heifers without clinical mastitis. Meanwhile, heifers without clinical mastitis had (OR ± SE) 3.0 ± 0.8 times greater odds of entering the parlor willingly (entry ease score = 1) ($X^2_{(1)}$ = 17.4, p < 0.01) compared to heifers with clinical mastitis.

4.4.2 Udder Health

Clinical mastitis and udder edema results are in presented in Table 4.3. All observed clinical mastitis cases were either mild (score 1) or moderate (score 2). Clinical mastitis was recorded at 13.9% of milkings on average. Over the first 3 DIM, approximately one-third (32.8%) of heifers developed signs of clinical mastitis and 13.4% had signs of udder edema. There was an effect of age on clinical mastitis, such that the odds of showing signs of clinical mastitis during the first 3 d of lactation decreased as calving age increased ($X^2_{(1)}$ = 8.4, p < 0.01).

There was an effect of treatment on quarter IMI and pathogens isolated (Table 4.4). Control heifer quarters had (OR ± SE) 5.4 ± 3.4 greater odds of being infected with *S. aureus*. Over a one-third (37.1%) of quarters and 15.8% of heifers were pathogen-free. *S. aureus* was the only contagious bacteria found in 9.4% of quarters. On average, the most common pathogen found was CNS, which was identified in 62 (39.0%) of quarters (*S. chromogenes* (22.0% of quarters), other non-*aureus Staphylococcus* spp. (15.1% of quarters), *S. sciuri* (1.3% of quarters) and *S. hominis* (0.6% of quarters)). Other pathogens were identified in 23 (14.5%) of quarters (*Bacillus* spp. (6.9% of quarters), *S. dysgalactiae* (1.9% of quarters), unidentified Gram-positive cocci (1.3% of quarters), *S. uberis* (1.3% of quarters), *E. cloacae* (0.6% of quarters), *E. faecalis* (0.6% of quarters), *E. coli* (0.6% of quarters), other non-*agalactiae Streptococcus* spp. (0.6% of quarters) and *Serratia* spp. (0.6% of quarters)).

Table 4.4. The probability (95% CI) of quarter intramammary infections (IMI) and pathogens isolated in milk within 36 hours after calving in heifers receiving a weekly 1.0% iodine-based teat dip in the parlor 3 weeks prior to calving (trained) or no treatment (control)

	Treatment		**Treatment effect [1]**		**Odds ratio (OR) ± SE**
Quarter IMI indicators, % probability	Train	Control	$X^2_{(1)}$	*p*-value	Train *vs* control
Heifers, *n*	34	23			
Quarters evaluated, *n*	98	61			
Quarters contaminated, *n*	33	30			
Quarter infection level [2]					
No growth	45.3 [34.4, 56.6]	34.8 [22.5, 49.6]	1.3	0.25	1.6 ± 0.6
Low	30.3 [21.3, 41.1]	23.7 [14.0, 37.2]	0.8	0.39	1.4 ± 0.5
Medium	7.3 [3.4, 15.0]	17.2 [8.4, 31.9]	3.1	0.08	0.4 ± 0.2
High	8.9 [4.0, 18.7]	16.7 [8.7, 29.5]	2.2	0.14	0.5 ± 0.2
Quarter pathogen isolate					
Staphylococcus aureus	4.2 [1.6, 10.7]	19.4 [10.0, 34.2]	8.1	<0.01	0.2 ± 0.1
CNS [3]	34.0 [24.0, 45.6]	29.5 [18.7, 43.3]	0.3	0.58	1.2 ± 0.5
Other pathogen [4]	12.4 [6.8, 21.4]	11.9 [5.4, 24.3]	0.0	0.93	1.1 ± 0.5

[1] Chi-square statistic of likelihood ratio test (LRT). [2] None = < 1; low = 1 to 10; medium = 11 to 50; high = > 50 CFU/0.1 mL. [3] Coagulase-negative staphylococci. Includes *Staphylococcus chromogenes*, *Staphylococcus hominis*, *Staphylococcus sciuri* and non-*aureus Staphylococcus* spp. [4] Includes *Streptococcus dysgalactiae*, *Streptococcus uberis*, non-*agalactiae Streptococcus* spp., *Bacillus* spp., *Enterobacter cloacae*, *Enterococcus faecalis*, *Escherichia coli*, *Serratia* spp. and unidentified Gram-positive cocci.

Herd and breed influenced some udder health outcomes. Quarters of conventional heifers had a greater probability of having no growth compared to organic (50.0 *vs* 30.6%; $X^2_{(1)} = 4.3$, $p = 0.04$). Quarters of organic heifers had a greater probability of being pathogen-positive at a high level compared to conventional (24.3 *vs* 5.7%; $X^2_{(1)} = 10.3$, $p < 0.01$). There was an effect of breed on quarter infection level, in which Holstein quarters had greater (p = 0.03) odds of high CFU growth compared to Normande, Jersey and Viking Red crossbred quarters, while Montbéliarde, Viking Red and Holstein crossbred quarters had similar ($p \geq 0.20$) odds of high CFU growth compared to Normande, Jersey and Viking Red crossbred and Holstein quarters ($X^2_{(2)} = 7.6$, $p = 0.02$).

4.5. Discussion

4.5.1. Behaviors

Trained heifers were less likely to kick and were more likely to be very calm during milking procedures, while control heifers were more likely to be very restless or hostile during milking procedures. These results indicate that the training program modulated aversive milking procedure behaviors to some extent. However, there were no effects of treatment on behaviors related to stomping, defecating, kicking off the milking unit and parlor entry ease. In a similar experiment, buffalo heifers that were habituated daily to milking parlor procedures for approximately 13 consecutive days before their expected calving date showed a reduction in the number of steps and kicks up to 20 days after calving [242]. Kutzer et al. [123] showed that pre-parturient training, which consisted of the introduction to the milking herd at least 10 days before calving and at least 10 visits to the parlor where milking staff provided tactile contact to the udder on the milking platform, reduced aversive behaviors in post-parturient heifers, such as stepping and kicking during milking procedures and tail clamping while entering the parlor. Das and Das [124] showed that at least 30 udder massage sessions 2 months prior to calving improved heifer temperament and reduced defecating during milking procedures on the first day of the lactation period. These previous experiments utilized a more rigorous training program than the present experiment, which employed weekly training for the 3 weeks leading up to parturition. Therefore, it is possible that the training regimen used in the present experiment was only sufficient in reducing kicks and improving milking procedure ease. Nevertheless, the training program used for the present experiment successfully modulated some aversive behaviors in heifers.

The training program used in this experiment represents a practical option for farmers that balances between modest and intensive training programs. For example, Ivemeyer et al. [140] reported heifers that received 4 handling sessions consisting of light touching on the neck by handlers twice per day on 2 separate days beginning 11 days before calving showed no difference in step or kick behaviors compared to non-handled heifers. On the contrary, intensive training programs requiring daily sessions consisting of passing through the milking parlor and receiving tactile contact at the udder, including washing, massaging and teat stripping, over approximately 13 to 14 days prior to parturition successfully improved heifer aversive behaviors [123,242]. Even though the training program used for the present experiment only required 3 sessions, it was sustained over a longer duration than described in previous experiments, which may explain why it successfully improved some aversive behaviors. For example, Boissy and Bouissou [243] suggested that additional handling may reduce the reactivity of heifers to humans when the handling program is sustained over a longer period of time (30 times spread out over 10 months) compared to intensive handling over a short period of time (30 times spread out over 3 months).

4.5.2. Udder Health

4.5.2.1. Clinical Mastitis

Contrary to our hypothesis, the development of clinical mastitis during the first 3 days of lactation was similar between treatments. This finding is similar to previous experiments that reported that iodine-based teat spraying or dipping of heifers during the last weeks before calving does not reduce early lactation clinical mastitis [137,144]. Approximately one-third of heifers showed signs of clinical mastitis over the first 3 days of lactation, which is similar to previous experiments, which reported that 22 to 23% of pasture-based heifers were diagnosed with clinical mastitis over the first 2 weeks of lactation [113,240]. Similarly, another experiment reported that 28% of the heifers developed signs of clinical mastitis during the first 5 days of lactation [144].

4.5.2.2. Quarter-Level Udder Health

Quarters of trained heifers were less likely to be infected with *S. aureus*, but overall intramammary infection levels were similar between treatments. These results suggest that the training regimen implemented for this experiment successfully reduced quarter-level *S. aureus* IMI. This finding is in agreement with previous experiments, which showed that spraying or dipping heifer teats during the last weeks before calving can reduce the prevalence of certain IMIs at calving [137,138]. For example, Lopez-Benavides et al. [137] reported that thrice-weekly application of iodine-based teat spray 3 weeks prior to calving reduced *S. uberis* but not CNS IMI. In contrast, Edinger et al. [144] found that teat dipping with 0.1% povidone iodine thrice-weekly for 3 weeks prior to calving did not reduce incidences of *S. aureus* or CNS IMI for heifers up to 5 DIM. The distribution and types of pathogens found in infected quarters is in agreement with results from previous experiments, which also reported that CNS was the most common bacteria isolated from post-parturient heifers [113,128,130,133,144,244–246].

4.5.2.3. Significance of *S. aureus*

Early lactation *S. aureus* IMIs are more likely to persist throughout lactation compared to other pathogen-specific IMIs and contribute to milk yield losses and increased somatic cell counts [116,247], proving to be one of the most difficult and important pathogens to control [248]. The estimated median duration of subclinical *S. aureus* infections is 64 and 91 days [249], and the likelihood of bacteriological cure is low [250]. Although *S. aureus* is generally lower in heifers compared to multiparous cows during early lactation [251], it can still be a major cause of clinical mastitis in heifers [114,252]. *S. aureus* is a contagious pathogen that is predominantly transmitted between herd mates [253] and quarters of the same cow [118]. However, infectious genotypes of *S. aureus* found in milk are also observed on the bodies of animals (e.g., skin and mucosal membranes) and in their environment [254,255].

In the present experiment, teat dipping heifers weekly 3 weeks before calving reduced quarter-level *S. aureus* IMI identified immediately after the termination of colostrum (within 36 hours after calving). The mechanism of action that pre-parturient teat dipping had on the reduction in post-parturient *S. aureus* IMI may be described by a variety of plausible explanations. It is possible that the teat dip prevented new *S. aureus* IMI during the treatment period. Up to 15% of quarters may be infected with *S. aureus* 1 to 3 weeks prior to calving [130–132,240,245,256]. Iodine teat dips at 0.1 to 0.5% have been shown to effectively prevent *S. aureus* IMI in lactating cows by 63.3 to 88.2% [257–259], so it is possible that that teat dip killed *S. aureus* on the teat end and prevented new *S. aureus* IMIs in pre-parturient heifers.

4.5.2.4. IMI Rate of Research Population

It appears that the population of heifers used for the present study had a higher rate of IMI than previous studies. For example, previous experiments reported that 48 to 91% of quarter samples taken within 5 days after calving had negative bacteriological culture results, which is greater than the 37% found for the present experiment [108,112,113,128,144,240,244]. Bludau [112] reported that 25% of heifers were non-infected 24 hours after calving, which is greater than the 16% found in the present experiment. Furthermore, the presence of *S. aureus* (9.4% of quarters) and CNS (39.0% of quarters) in quarters for the present experiment are much higher than many previously reported values, in which previous experiments reported that approximately 0.6 to 2.7% and 4.8 to 9.7% of quarters were infected with *S. aureus* and CNS within 5 days after calving, respectively [108,113,135,240]. On the contrary, a few experiments reported comparable intramammary infection rates within 48 hours after calving for *S. aureus* (3.9 to 10.9% of quarters) and CNS (33.2 to 47.3% of quarters) [145,246]. It is possible that herd-level specifics and farm management contributed to the high level of IMI in the population of heifers used in the present experiment.

The high level of IMI found in the present experiment may be greater than previous reports due to atypical housing and management, such as pasture housing and a lack of antibiotic therapies. For example, pastured heifers may have had an increased risk of *S. aureus* IMI due to biting flies transferring the pathogen between infected and non-infected animals [260]. Based on previous experiments using the same research herd [19], it is possible that stable flies (*Stomoxys calcitrans* L.) and horn flies (*Haematobia irritans* L.) may be a contributing factor to the spread of *S. aureus* within the herd. Furthermore, the outdoor housing used in the current experiment could have increased the risk of elevated IMI, mainly due to unhygienic legs and udders caused by heavy precipitation events followed by wet and muddy surroundings [240,261].

4.5.2.5. Other Effects on Udder Health

Pathogen-positive quarters at a high CFU level were more commonly observed in quarters of organic heifers and of Holstein heifers. Similarly, Persson Waller et al. [136] reported lower somatic cell counts in conventional heifers compared to organic heifers over the first 2 milkings of the lactation period. For the present experiment, it is possible that differences between conventional and organic post-parturient housing and genetic differences played a major role in the development of IMI and clinical mastitis. Previous experiments reported that unhygienic legs and udders may put cows at risk for clinical and subclinical mastitis due to environmental pathogens [240,261]. Furthermore, Persson Waller et al. [114] also reported a possible effect of breed on udder health, indicating that breed may play a role in susceptibility to mastitis.

4.5.3. Limitations

Although the training program was associated with positive effects on animal behavior and udder health in the present experiment, the limited number of animals and fairly high incidence of clinical mastitis will need to be considered before generalization of the results. Producers should consider how differences in animals, management and environment could play a role in the application of these results. For example, heifer temperament [106,107] and milking parlor type [139] may influence the behavioral outcomes of this training protocol. Likewise, herd-specific factors, such as the current level of IMI, may influence outcomes of teat dipping pre-parturient heifers, such that improvements may be unapparent or more apparent if certain pathogens are present or absent [251]. Therefore, future investigations of aversive behavior and mastitis prevention strategies using methods such as those of the present experiment should be investigated under a variety of management systems.

4.6. Conclusions

Teat dipping pre-parturient heifers in the milking parlor weekly beginning 3 weeks before calving reduced the occurrence of some aversive behaviors and the risk of *S. aureus* IMI in early lactation heifers. Such treatment led to improved milking ease scores and reduced kicks during milking procedures over the first 3 days of the lactation period. However, pre-parturient treatment was not associated with significantly improved parlor entry ease scores nor reduced stomping, defecating, or kicking off milking units. The pre-parturient treatment resulted in reduced *S. aureus* IMI observed immediately after the collection of colostrum (within 36 hours of calving). Yet, the development of clinical mastitis and udder edema in heifers over the first 3 days of the lactation period was not affected by pre-parturient treatment. Likewise, pre-parturient treatment did not markedly reduce overall quarter IMI and resulted in comparable quarter IMI caused by CNS and other environmentally transmitted pathogens. Therefore, the results from this experiment suggest that weekly teat dipping 3 weeks before the expected calving date may modulate some aversive behaviors and *S. aureus* IMI in early lactation heifers.

4.7. Publisher and Collaborator Recognition

A modified version of this chapter was accepted for publication in *Animals* on 2021 May 31 [262]. I would like to express my gratitude to Ulrike Sorge and Bradley Heins for their contributions to this study and co-authorships for this publication. I would also like to express my gratitude to the staff at the West Central Research and Outreach Center for their assistance in data collection and care of animals. This work is supported by Organic Agriculture Research and Extension Initiative (OREI) [grant no. 2016-51300-25734/project accession no. 1010693] from the USDA National Institute of Food and Agriculture.

Chapter 5: Efficacy of Broilers as A Method of Face Fly (*Musca autumnalis* De Geer) Larva Control for Organic Dairy Production

5.1. Summary

The objective of this study was to evaluate Freedom-Ranger broiler chickens as a method to control face fly (*Musca autumnalis* De Geer) larvae in cow dung pats on pasture. Ninety-nine pats in 3 replicates were inoculated with first-instar larvae and exposed to one of four treatment conditions for 3 to 4 days: 1) an environment-controlled greenhouse (GH); 2) pasture without broilers (NEG); 3) pasture with 25 broilers stocked at a low density of 2.5 m^2 of outdoor area per broiler (LOW); and 4) pasture with 25 broilers stocked at a high density of 0.5 m^2 of outdoor area per broiler (HIGH). Broiler behaviors and weather conditions were recorded twice daily. Survival rates of larvae (mean, 95% CI) were similar for pats in the NEG (4.4%, 2 to 9%), LOW (5.6%, 3 to 11%) and HIGH (3.2%, 2 to 7%) groups, and was greatest for larvae reared in the GH (54.4%, 36 to 72%) group compared to all other groups. The proportion of broilers observed pasture ranging was 14.0% (6 to 28%) but was negatively related to solar radiation. Broilers were never observed foraging in pats. Results indicate that use of broilers may not be an effective method for controlling larvae of dung pat breeding flies.

Keywords: organic, animal welfare, broiler chickens, dairy cattle, pest management, behavior, pasture, face fly, dung fly, fly control

5.2. Introduction

Flies are considered an important animal welfare concern due to the negative effects flies may have on the affective state, behavior and health of organic dairy cattle [12]. In a review of dairy industry changes that affect animal welfare, Barkema et al. [76] suggested that future research should begin classifying effective and ineffective organic-approved fly management strategies. Research surveying organic dairy producers and veterinarians in the US acknowledged that the lack of scientific support for certain practices utilized by organic livestock producers jeopardizes animal welfare [27,75]. Alternative management strategies used in organic livestock production require support from controlled research trials to confirm that the strategies indeed improve animal welfare.

Little is known about broiler behaviors in relation to interacting with cow manure, such as movement on pasture, time budgets and foraging from dung pats or elsewhere. The consumption of pasture contents, including forages, insects and larvae, may be affected by weather and stocking density [263]. Therefore, it is critical to assess behaviors and factors affecting behaviors to support findings of studies that depend on consumption of pasture contents. The objectives of this study were: 1) to determine if broiler chickens affect the survival rate of face fly larvae presented in cow manure pats on pasture and 2) to assess broiler pasture ranging and behaviors and their responses to weather conditions.

5.3. Materials and Methods

5.3.1. Animal Care and Housing

The University of Minnesota Institutional Animal Care and Use Committee approved all animal care and procedures specific to this experiment (protocol number 1607-33960A).

The experiment was conducted from June to August 2018 at the West Central Research and Outreach Center (Morris, MN) in pastures that were consecutively grazed by lactating dairy cows (*Bos taurus* L.) and broiler chickens (*Gallus gallus domesticus* L.). The dairy herd and pastureland used in this study had been certified organic since 2010 by the Midwest Organic Services Association, following regulations set forth by the United States Department of Agriculture (USDA) National Organic Program (NOP). Cows in this study were housed on pasture for 22 hours per day and spent the remaining 2 hours per day for milking procedures which took place twice daily in a swing-nine para-bone milking parlor at 06:00 and 17:00. A total of 80 cows grazed the pastures used in this study, which were rotationally stocked at a rate of 4 cows per hectare. Cows rotated to a new paddock every 2 days based on forage biomass availability. Pastures included perennial forbs, grasses, and legumes, such as alfalfa (*Medicago sativa* L.), chicory (*Cichorium intybus* L.), meadow brome grass (*Bromus riparius Rehmann*), meadow fescue (*Schedonorus pratensis (Huds.) P. Beauv*), orchard grass (*Dactylis glomerata* L.), perennial ryegrass (*Lolium perenne* L.), red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens*

L.). Cows had ad libitum access to minerals and water and were supplemented with 2.72 kg of organic corn grain daily.

The study involved a total of 150 mixed-sex Freedom Ranger broiler chickens (Welp Hatchery, Bancroft, IA) in 3 replicate groups of 50 that hatched on 1 May, 29 May, and 9 July, respectively. The Freedom Ranger hybrid consisted of a four-line cross developed in the 1960's to meet the French Label Rouge Free Range program standards. For each replicate group, day-old broiler chicks, vaccinated for Marek's at hatch, were housed in a 2.22 m^2 pen bedded with 5 cm of wood shavings and had *ad libitum* access to feed and water. Broilers in each group began acclimating to experimental housing at 4 weeks of age and were randomly assigned to pens with 2.5 m^2 or 0.5 m^2 of adjacent pasture per bird. These stocking densities were chosen based on Humane Farm Animal Care and American Humane Farm Program animal welfare standards for "Free-Range" and "Pasture-Raised" chickens [172,264]. Pens were balanced by bird sex and weight and housed 25 broilers in each half of a floorless 3.7 × 3.7 m mobile shelter (Chicken Ranger Coops, Narvon, PA) that was divided into 2 equally sized areas (1.8 × 3.7 m). One door (0.91 × 0.91 m) per pen allowed broilers free choice between the shelter and pasture during the day, and confinement to the shelter at night to protect the birds from predators. The perimeters of the pens were fenced with 1.2-m tall portable electric poultry netting (PoultryNet, Premier1Supplies, Washington, IA) that was continuously charged by a solar powered 0.60 joule energizer (IntelliShock, Premier1Supplies, Washington, IA).

For the duration of the study, each group of broilers had ad libitum access to water and granite grit and were fed a restricted diet of 113 g of concentrate per bird (20% crude protein; Chick Starter AMP, Vita Plus Corporation, Madison, WI) daily at dusk (between 21:00 and 22:00) prior to shelter confinement and removed between 05:00 and 06:00 the next morning to encourage pasture foraging. The health of each bird was assessed prior to study initiation, starting at 4 weeks of age and weekly thereafter. Hock lesions and foot pad lesions were assessed during each weekly heath assessment. No broilers had hock or foot pad lesions or showed signs of gait difficulties.

5.3.2. Experimental Design

This study was conducted as a randomized complete block design with repeated sampling, blocked by replicate group. On each block’s day of treatment initiation (21 June, 24 July, and 15 August), fresh dung was collected between 05:00 and 06:00 from randomly selected cows in neighboring paddocks, homogenized by hand mixing, and stored in a covered bucket until use the same morning. Inocula for dung pats were prepared by counting and transferring aliquots of 100 first-instar face fly (*M. autumnalis*) larvae into 5 g of dung in each of 33 covered Petri dishes. Larvae were obtained from a lab colony. Petri dishes and their contents were stored at 23 °C to delay development until treatment assignment. At 12:00, one-liter dung pats (33 per replicate group; 99 total) were consecutively deposited in treatment conditions and inoculated with maggots by random assignment among the four treatment conditions: 1) on 8 cm of sand in 5-L buckets in a naturally ventilated environment-controlled greenhouse (GH, n = 9 pats); 2) on pasture without broilers (NEG, n = 30 pats); 3) on pasture with broilers stocked at a low density (LOW, n = 30 pats); or 4) on pasture with broilers stocked at a high density (HIGH; n = 30 pats).

Dung pats were placed equal distances apart in the outdoor portion of the broiler pens for the LOW (10 pats 0.9 m apart) and HIGH (10 pats 0.5 m apart) treatment groups, and adjacent to the broiler pens for the NEG treatment group (10 pats 0.9 m apart). Pats were inoculated by each Petri dish into the center of a recipient pat. Treatment within replicate served as the experimental unit (3 replicates of 4 treatments per rep = 12) and pat within experimental unit was treated as a sub-sample. Over the 3 replicates, average (± SD) broiler age was 48 ± 10 days and average broiler weight was 1.94 ± 0.7 kg at the onset of the experiment.

5.3.3. Data Collection

Once inspections indicated the transplanted larvae reached the third-instar, after 3 or 4 days, pats and the 3 cm of soil underneath pats in the NEG, LOW, and HIGH treatment groups were transferred to 5-L buckets with 8 cm of sand. The buckets were housed in the greenhouse neighboring the buckets housing pats of the GH treatment group until the larvae began to pupate after 3 to 5 days. The number of larvae and pupae were then counted in each bucket by wet sieving sand and pats through a 1.41 × 1.41 mm square wire mesh sieve to extract surviving larvae and pupae.

Behavior observations were recorded by an observer for the second and third replicates of the study in the morning (between 09:00 and 11:00) and afternoon (between 13:00 and 17:00) when precipitation was not expected for a total of 2 observation periods per day. Prior to behavior observations, pasture ranging was recorded for each pen as the proportion of broilers outside of the shelter. Behaviors were then recorded in continuous 60-second observation periods on 10 individual focal broilers per pen using the Animal Behaviour Pro mobile app (version 1.2) [265]. Focal broilers were identified using livestock paint and were observed in random order alternating between treatment pens. Behavioral states corresponding to the time budget were recorded as durations and foraging events were recorded as binary outcomes (i.e., the occurrence of foraging behaviors within the 60-second observation period was recorded as either a yes [presence] or no [absence]). The frequency of foraging bouts was not recorded. An ethogram defining recorded behaviors is in Table 5.1.

Table 5.1. Ethogram of behaviors recorded before and during 60-second observation periods. Modified from Ventura et al. [266].

Behavior	Definition
Pasture ranging	Proportion of flock outside the shelter before start of behavior observation
Time budget [1]	
Sit	Bird has its breast in contact with the ground. Eyes are open
Stand	Bird maintains upright position on its extended, stationary legs
Sleep	Bird has its breast in contact with the ground. Eyes are closed
Travel	Bird is displaced on the ground, in which the action of legs propels the bird
Foraging [2]	
Ground foraging	Bird pecks or scratches at the ground
Pat foraging	Bird pecks or scratches at dung pat

[1] Time budget behaviors are mutually exclusive. Recorded as duration. [2] Foraging behaviors are non-mutually exclusive, and foraging behaviors and time budget behaviors are non-mutually exclusive. Recorded as binary outcomes.

The University of Minnesota West Central Research and Outreach Center weather station recorded measures of ambient humidity, ambient temperature, precipitation, solar radiation, and wind speed every 15 minutes. The comprehensive climate index (CCI) was calculated to describe the apparent temperature based on ambient humidity, ambient temperature, solar radiation and wind speed variables [267]. For each pasture ranging and behavioral observation, the time was rounded to the nearest 15-minute interval and matched with the climatic condition data.

5.3.4. Statistical Analysis

Logistic regression models with beta error distributions and logit link functions were used to analyze 7 binomial outcomes for larval survival, and broiler pasture ranging and behaviors. Modeling was accomplished in RStudio (version 1.3.1073) [268] using the *glmmTMB* package [197]. All models included fixed effects of treatment and replicate, and a random effect of treatment within replicate to account for the dependency among repeated sampling within experimental units.

Nonparametric correlation coefficients, Spearman's rho, were used to examine pair-wise relationships between pasture ranging and behaviors and weather conditions at each time of observation. Stocking density treatment could not be included as an independent variable in correlations, so each correlation was initially performed on each stocking density treatment and replicate; there were no differences in direction or *p*-values between stocking density. Therefore, observations were pooled, and the correlation coefficient is reported with the corresponding degrees of freedom (df).

The one weather variable with the strongest correlation with the chosen dependent variable was included in the models for pasture ranging and behaviors. For the analysis of pasture ranging, a fixed covariate of *solar radiation* was included. The analyses for behaviors of the time budget included a fixed covariate of *CCI* and a random effect of *broiler* to account for repeated sampling. For the analysis of foraging events, pen averages were taken to represent the proportion of broilers observed performing the behavioral event for each observation and no covariate was included in the model based on the absence of a relationship between foraging and climatic conditions.

Type II Wald χ^2 tests were used to test the significance of main effects and are reported with the corresponding degrees of freedom followed by number of observations. The Tukey adjustment was applied to compare groups when the corresponding main effect had $p \leq 0.05$. Marginal mean rates and 95% confidence intervals (CI) for all responses were transformed to the natural scale. Treatment groups were compared using rate ratios (RR), the ratio of mean rates between two groups.

5.4. Results

5.4.1. Climatic Condition and Precipitation

Daily weather conditions over the course of the experiment are displayed in Figure 5.1. Study replicates occurred from 21 to 25 June (replicate 1), 24 to 27 July (replicate 2), and 15 to 19 August (replicate 3). The average ambient temperature recorded during study replicates 1, 2 and 3 were 22.3 °C, 19.0 °C and 21.4 °C, respectively. The ambient temperature range (minimum to maximum) recorded during study replicates 1, 2, and 3 were 15.0 to 28.9, 11.7 to 28.3 and 15.0 to 28.3 °C, respectively. Total precipitations accumulated during study replicates 1, 2 and 3 were 7.1, 9.7, and 0.0 mm, respectively. Precipitation during study replicates 1 and 2 took place on 24 June from 02:45 to 03:00 and on 25 July from 04:00 to 07:00, respectively.

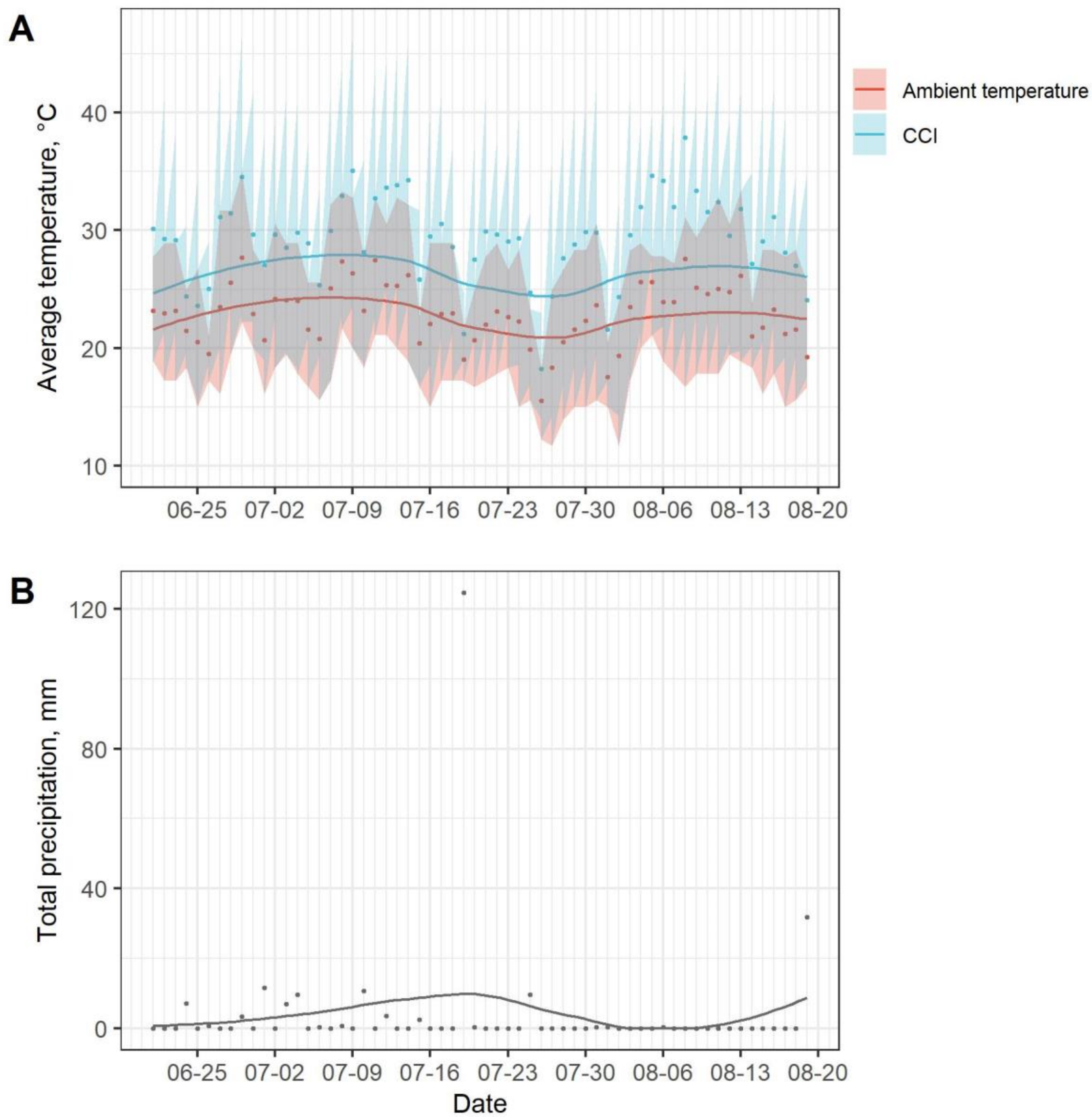

Figure 5.1. Daily weather during experiment. A) Average daily ambient temperature and average daily comprehensive climate index (CCI), apparent temperature. Transparent bands represent daily minimum and maximum values. B) Total daily precipitation.

The means (range) for CCI, ambient humidity, ambient temperature, solar radiation, and wind speed recorded during observations were 30.7 °C (15 to 40 °C), 73% (57 to 87%), 23.2 °C (16 to 28 °C), 415 W/m^2 (64 to 679 W/m^2), and 0.74 m/second (0 to 1.8 m/second), respectively.

5.4.2. Face Fly Larva Survival

Larval survival rates by treatment are shown in Figure 5.2. Upon transferring pats from the pasture, evidence of trampling but not scratching nor pecking was apparent for almost every pat in the broiler treatment groups (LOW and HIGH), but not for the pasture control group without broilers (NEG). There was an effect of treatment on the survival rate of larvae ($\chi^2_{(df = 3, n = 98)} = 55.2$, $p < 0.01$).

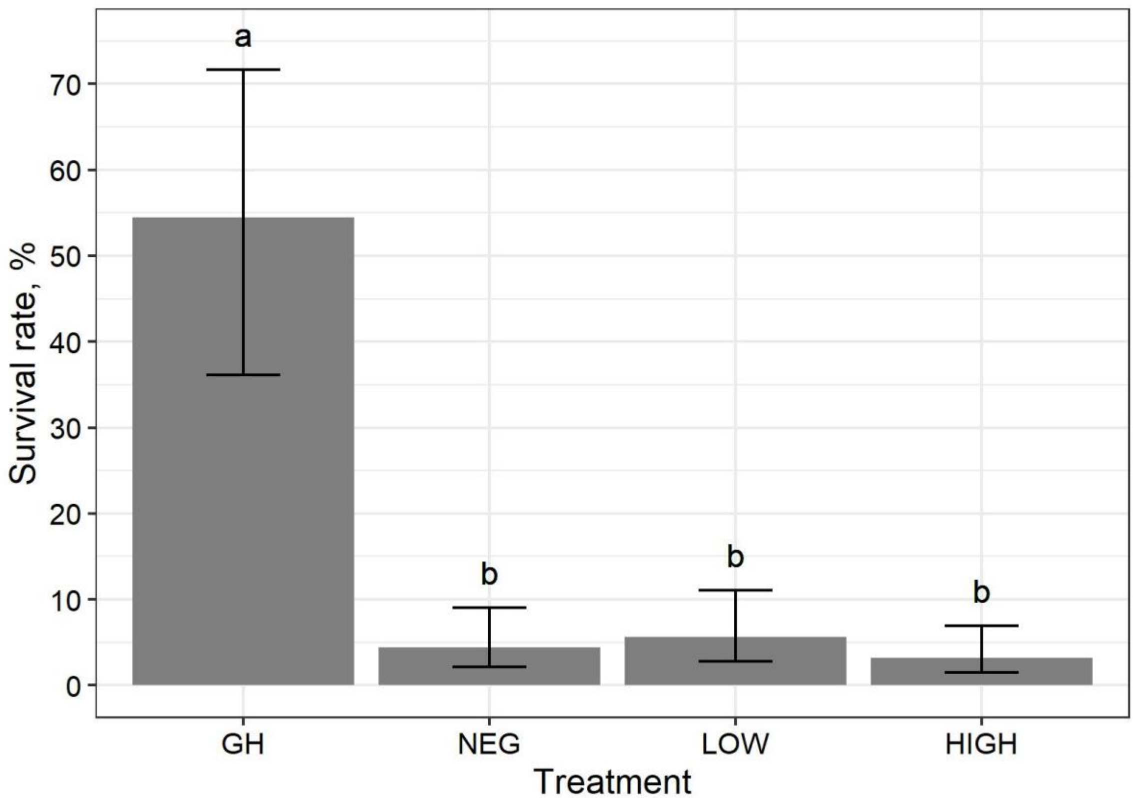

Figure 5.2. Mean survival rates (± 95% CI) of face fly (*Musca autumnalis* De Geer) larva reared in cow dung pats under different treatment conditions. Treatment means with a different letter are different at $p \leq 0.05$. Treatments: GH = greenhouse; NEG = on pasture without broilers; LOW = on pasture with low density broilers (2.5 m^2 outdoor area per bird); HIGH = on pasture with high density broilers (0.5 m^2 outdoor area per bird).

For the effect of treatment, the survival rates (95% CI) were 54.4% (36 to 72%), 4.4% (2 to 9%), 5.6% (3 to 11%), and 3.2% (2 to 7%) for pats in the GH, NEG, LOW, and HIGH groups, respectively. The survival rate for larvae in the GH treatment group was greater (RR, 95% CI) compared to the NEG (11.9, 4 to 34), LOW (9.4, 3 to 26) and HIGH (16.3, 5 to 49) treatment groups ($p < 0.01$). The NEG, LOW, and HIGH treatment groups had similar survival rates ($p > 0.70$).

5.4.3. Pasture Ranging

There was an effect of solar radiation on pasture ranging, such that ranging decreased as solar radiation increased ($\chi^2_{(df = 1, n = 22)} = 8.9$, $p < 0.01$). There was no effect of stocking density on pasture ranging. On average, only a small proportion of the flock was observed pasture ranging (mean = 14.0%, 95% CI = 6 to 28%). Furthermore, no birds were observed pasture ranging for over a third (36%) of the observations. Results for the analysis of pasture ranging indicate that broilers are less likely to range in the pasture during periods of high solar radiation regardless of stocking density treatment and suggest that overall pasture use is low for the experimental conditions of the study, which were characterized by open cattle grazing pasture and lack of tree cover.

Correlations between pasture ranging and other climatic conditions recorded during observations are shown in Table 5.2. The correlations indicate a negative relationship between pasture ranging and CCI and ambient temperature, and a positive relationship between pasture ranging and ambient humidity. Excessive heat and solar intensity appear to be important influencers of pasture ranging in broilers. These results suggest that the hybrid broilers used in this study under these experimental conditions characterized by no tree cover avoid overheating by reducing activity in sun and seek shade as levels of heat stress increase.

Table 5.2. Spearman's rank correlation coefficients and *p*-values for relationships between broiler pasture ranging, time budget behaviors, and ground foraging with climatic conditions

Behavior (df) [1]	CCI [2]	Temperature	Humidity	Solar radiation	Wind speed
Pasture ranging (20)	−0.43 (0.05)	−0.54 (0.01)	0.53 (0.01)	−0.68 (<0.01)	−0.13 (0.55)
Time budget (197)					
Sit	0.17 (0.02)	0.18 (<0.01)	−0.10 (0.17)	0.13 (0.06)	−0.05 (0.46)
Stand	0.04 (0.56)	−0.01 (0.94)	−0.10 (0.18)	0.01 (0.87)	−0.01 (0.89)
Sleep	−0.17 (0.02)	−0.14 (0.04)	0.16 (0.02)	−0.14 (0.05)	0.06 (0.42)
Travel	0.13 (0.06)	0.05 (0.47)	−0.03 (0.67)	0.05 (0.72)	−0.20 (<0.01)
Ground foraging (18)	0.16 (0.51)	0.17 (0.47)	−0.31 (0.19)	0.10 (0.69)	0.30 (0.19)

[1] df = degrees of freedom for Spearman's rank correlation test. [2] CCI = comprehensive climate index, apparent temperature.

5.4.4. Time Budget

The time budget estimates for the effect of CCI are visualized in Figure 5.3. For the analyses of time budget behaviors, there was no effect of stocking density treatment on any time budget behavior. The effect of CCI only existed for the analyses of standing ($\chi^2_{(df = 1, n = 199)}$ = 6.2, p = 0.01) and sleeping ($\chi^2_{(df = 1, n = 199)}$ = 4.6, p = 0.03). On average, the time budget (95% CI) was comprised of 41.4% (31 to 52%) sitting, 30.1% (20 to 43%) standing, 14.1% (8 to 24%) sleeping, and 1.4% (1 to 3%) traveling.

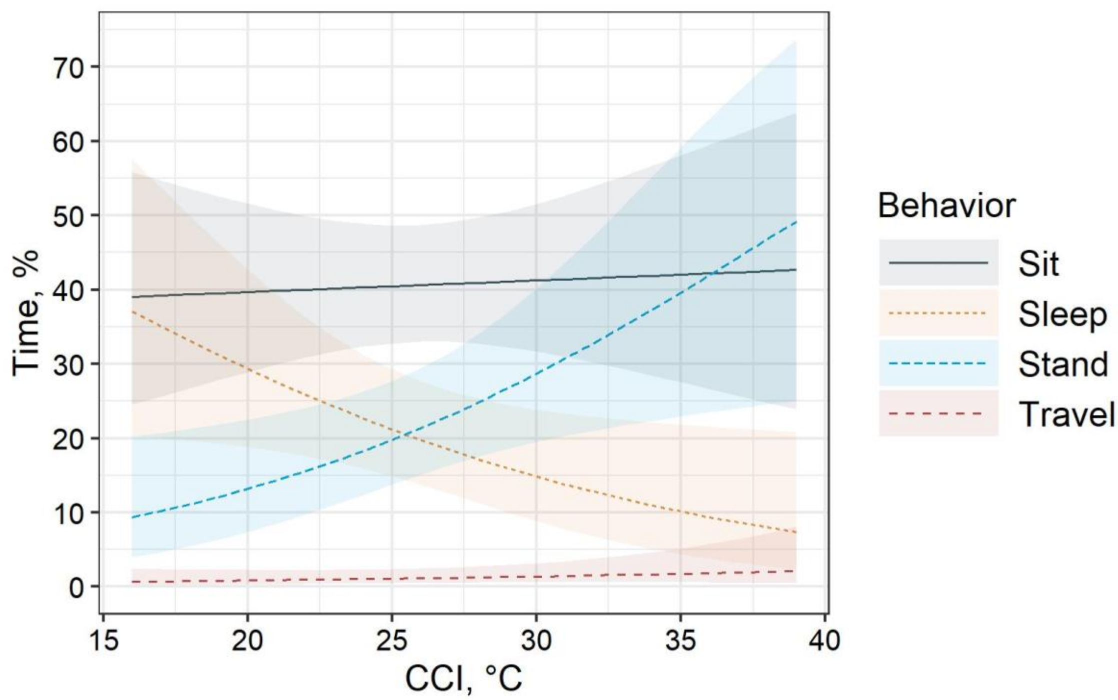

Figure 5.3. Mean percentages of time (± 95% CI) broilers performed time budget behaviors (sitting, sleeping, standing, and traveling) for the effect of CCI. The effect of CCI had p = 0.01 and p = 0.03 for the analyses of standing and sleeping, respectively. CCI = comprehensive climate index, apparent temperature.

The time budget analyses indicated that for every 1 °C increase in CCI, the proportion of time broilers were observed standing increased by a factor of

1.10 (regression coefficient = 0.10, 95% CI = 0.02 to 0.17). Alternatively, the proportion of time broilers were observed sleeping decreased by a factor of 0.92 for every 1 °C increase in CCI (regression coefficient = −0.09, 95% CI = −0.17 to −0.01). These results indicate that increased CCI values between 16 and 39 °C may disrupt sleeping and standing behaviors.

Correlations between time budget behaviors and other climatic conditions recorded during observations are shown in Table 5.2. In general, all correlations indicated weak strengths of association. Sitting had positive relationships with ambient temperature and solar radiation, whereas sleeping had negative relationships with the same noted climatic conditions. Sleeping also had a positive association with ambient humidity. Traveling had a negative association with wind speed. Sleeping appeared to be the most affected by climatic conditions based on the correlation coefficients.

5.4.5. Foraging

For the analysis of ground foraging, there was no effect of stocking density treatment. On average, the percentage of broilers recorded ground foraging during observations was 21.5% (95% CI = 6 to 54%). At least one broiler ground foraged during 90% of observations. Broilers were never observed pat foraging, but predation of insects and frogs was observed during ground foraging events.

Correlations between ground foraging and climatic conditions recorded during observations indicated that ground foraging was not associated with any climatic condition (Table 5.2).

5.5. Discussion

This study is the first to our knowledge to specifically examine predation on dung fly larvae by pastured poultry. Freedom Ranger broilers had no effect on survival rates of face fly larvae. Related studies on the utilization of poultry as fly predators reported that pastured laying hens were successful at managing weed seeds [269]. Furthermore, Muscovy ducks (*Cairina moschata* L.) successfully consumed and reduced populations of house fly (*Musca domestica* L.) larvae and adults in closed calf rooms, but not in open maternity pens [270,271]. Another study showed that 6-week old Barred Plymouth Rock chickens (*Gallus gallus domesticus*) and African geese (*Anser cygnoides* L.) reduced the number of insect pests in an apple orchard intercropped with potato crops [272]. Under further investigation, this study also reported that muscid (Muscidae) larvae and adults were found in 0% and 33 to 40% of chicken crops, respectively [272]. A possible limitation of the current study is that transferring pats from the pasture to the greenhouse prior to larva pupation may have limited the potential for broilers to prey on pupae, but there were few larva survivors anyway, so this artifact was likely small. Results from previous research [270–272] agrees with the results of the current study, which indicate that broilers may not be successful predators of dung fly larvae that pass their developmental stages in cattle manure.

It is unknown whether face fly larvae are truly an attractive feed source for broilers. Anecdotal evidence suggests that face fly larvae and pupae are highly sought after when they are hand-fed to broilers. In an early study, Dashefsky et al. [273] successfully fed face fly pupae to day-old White Rock chickens, yet the level of palpability was not determined or discussed. Other studies [274–277] suggested that black soldier fly (*Hermethia illucens* L.), house fly (*Musca domestica* L.) and yellow mealworm (*Tenebrio molitor* L.) larvae can successfully be used as a palatable feed supplement for poultry. Although research on the palatability of face fly larvae for poultry is sparse, insect larvae in general is a highly attractive feed source for poultry.

The Freedom Ranger broilers used in the current study were a hybrid cross of several strains and were originally bred for their suitability for pasture housing systems. Previous studies [162,278] suggest that hybrid strains that demonstrate slower growth and improved mobility compared to pure strains make hybrids more equipped for free-range systems. A study by Lambertz et al. [278] demonstrated that hybrid male broilers (Bresse-Gauloise × New Hampshire) grew faster but had similar carcass quality to their purebred equivalent (Bresse-Gauloise). Other slow-growing hybrid broilers (Labresse × L86) were previously demonstrated to have increased pasture ranging, improved gait scores, and reduced dermal lesions compared to their fast-growing counterpart (Ross 208) [162]. Alternatively, another study [279] reported that a slow-growing pure strain (White Bresse L40) demonstrated increased pasture ranging and foraging behaviors compared to a fast-growing hybrid strain (Kosmos 8 Red), suggesting that speed of growth may be an important influencer of broiler behavior.

Stark differences in larvae survival rates were apparent between pats reared in the pasture treatment groups (NEG, LOW, and HIGH) and those raised in the greenhouse (GH). Valiela [161] also reported lower face fly larvae survival rates when reared in field conditions compared to laboratory conditions for the first 5 days after hatching, in which the majority of morality occurred on the first day of field exposure. Results from the current study are similar to those of the experiments conducted in this previous study [161], which indicate that pasture conditions dramatically increase the mortality of face fly larvae.

The overall survival rates of larvae in this study were lower than previous reports. Valiela [161] reported greater mean survival rates of 78 to 76% and 42 to 31% after 3 to 5 days of exposure to laboratory conditions maintained at a constant temperature of 32 °C and field conditions, respectively. The reduced survival of face fly larvae of the current study may have been caused by several factors, including temperature and predation.

Cool temperatures may have increased the mortality of face fly larvae for the current study. First-instar larvae are especially prone to temperature extremes since their mobile abilities are not fully developed. In an experiment on the survival of face fly first-instar larvae, Valiela [161] found that constant temperatures of 35 to 40 °C maximized the survival rate at 69%, whereas temperatures of 10, 16 and 20 °C yielded survival rates of 0, 22 and 53%, respectively. This study reported mortality rates of 1 to 2% per hour at constant temperatures from 10 to 20 °C [161]. Within the first 24 hours of pat placement for the current study, temperatures of ≤ 16 °C were not observed; however, temperatures of ≤ 20 °C were observed in single durations of 8, 10 and 8 hours for replicates 1, 2 and 3 of the study, respectively. Therefore, low temperatures may have accounted for 8 to 20% of face fly larvae mortality in pasture treatment groups. Alternatively, the greenhouse environment may have provided a more temperate environment conducive for better early life face fly larvae survival, as it was protected from extreme temperature fluctuations. Yet, the survival rate of face fly larvae reared in the greenhouse was lower than reported in previous studies where larvae were reared in cow pats under laboratory conditions. Although the greenhouse may have somewhat shielded pats from extreme temperature fluctuations, it is still possible that temperatures dropped below 20 °C, resulting in larvae mortality. Therefore, cool temperatures observed during the current study may have reduced the survival of face fly larvae in all treatment groups.

Naturally occurring predatory arthropods may have consumed some of the face fly larvae. It is possible that dung beetle (*Sphaeridium* spp.) larvae and rove beetles (*Philonthus cruentatus* Gmelin) preyed upon face fly larvae [280,281]. A study conducted at a prairie cattle pasture confirmed the presence of dung beetles (*S. lunatum* Fabricius and *S. scarabaeoid* L.) and rove beetles (*P. cruentatus*) in Minnesota [282]. For the current study, there was evidence of beetle tunneling for every undisturbed pat in the pasture treatment groups, suggesting the presence of predatory beetles. In a study to investigate the arthropod predation on face fly larvae, Valiela [161] found that the introduction of dung beetles (*S. scarabaeoid*) and rove beetles (*P. cruentatus*) reduced the larvae survival rates by 14 and 57%, respectively. Moreover, the presence of both predators reduced the larvae survival rate by 66% [161]. Based on this information, it is possible that naturally occurring dung beetles and rove beetles preyed on face fly larvae of the current study, which would explain the majority of the survival differences among pats raised in the greenhouse and pats raised in the pasture.

It is also possible that the cattle dung use in this study reduced the survival of fly larvae due to the presence of alkaloids. A recent study by Parra et al. [283] reported that the dung from cattle that consumed endophyte-infected fescue contained two important alkaloids, peramine and lolitrem B, which reduced horn fly (*H. irritans*) larval survival from 45 to 10%. Based on this information, it is possible that the consumption of perennial ryegrass (*L. perenne*) included in the pastures of the current study resulted in the presence of these alkaloids and consequently lowered the survival of the fly larvae.

A fundamental necessity for the successful utilization of broilers as a method of controlling dung fly immature development is pasture ranging. For the current study, only an average of 14.0% of broilers were observed ranging, which was negatively affected by increasing solar radiation. Previous studies using slow-growing strains (Delaware, Labresse × L86, and Sherwood White) similarly reported that 9 to 25% of broilers were observed ranging [162–164] and that 34 to 38% of observations resulted in lack of ranging [164]. Stadig et al. [166] similarly reported that increased solar radiation had a negative effect on slow-growing broiler (Sasso T451) ranging for values of 0 to 1000 W/m^2. Hegelund et al. [284] reported a negative relationship between laying hen (ISA Brown and Lohmann Brown) pasture ranging and ambient temperatures of 17 to 41 °C, which agrees with correlation results of the current study. The low pasture ranging observed in this study may also be partially explained by an undesirable pasture habitat. It is intuitive that chickens prefer covered areas since their domestication evolved as descendants of Red Jungle Fowl (*Gallus gallus* L.), which rely on vegetative cover for protection from predators [285]. Since the pasture space of the current study was open and comprised of forages that were 9 to 11 cm tall, it was not surprising that broilers of this study were commonly observed inside the shelter where safety from solar radiation and predators could be preserved. It is possible that a more temperate climate with cooler summers would help promote pasture ranging in a similar treeless pasture habitat used for cattle grazing. However, cattle pastures without trees or shelters would still be unsuited for promoting pasture ranging even under optimal weather conditions since they do not provide protection from predators.

Outdoor structural enrichments providing cover may make pasture ranging more desirable to broilers by filtering solar radiation that causes excessive heat [164] and providing a sense of protection from aerial predators [285]. For example, canopy enrichments were previously demonstrated to improve range utilization in areas up to 20 to 31 m from the shelter [164,178]. Dawkins et al. [163] reported that slow-growing broilers (Sherwood White) preferred ranging in areas with tree cover as opposed to areas with short grass in areas 10 m from the shelter. Stadig et al. [166] found that more slow-growing hybrid broilers (Sasso T451) left the shelter and ranged more than 5 m from the shelter when provided tree cover compared to artificial cover. However, studies conducted by Dawkins et al. [163] and Fanatico et al. [164] similarly concluded that slow-growing broilers (Delaware and Sherwood White) are reluctant to leave their shelter even when offered cover in the pasture area. Although access to pasture may be plentiful, broilers realistically spent the majority of their time in the shelter and away from any potential opportunities to forage for dung fly larvae in cow pats.

Behavioral observations supplement the face fly larvae survival findings of the current study. Broilers were never observed foraging in the dung pats (though they may have done so during non-observed periods). In fact, broilers were observed to spend most of their time sitting. Fanatico et al. [164] also reported that sitting was more commonly observed than standing or walking in 7- and 10-week-old slow-growing Delaware broilers with access to pasture. The time spent standing and sleeping was associated with climatic conditions. Furthermore, only about 22% of broilers were observed performing ground foraging behaviors during observations. In agreement with the current study, Fanatico et al. [164] similarly observed foraging for 28% of observations when averaged across age groups and pen locations for slow-growing Delaware broilers. A possible limitation of this study includes infrequent behavioral sampling. Continuous sampling from dusk until dawn would provide a more accurate estimate of behaviors.

It is unknown whether or not broilers of an age group older than that used in the current study (approximately 7-weeks-old) would have yielded different study results. Fanatico et al. [164] reported that outdoor foraging events increased by a factor of 1.7 between 7 and 10 week-old slow-growing broilers. Almeida et al. [279] similarly reported that outdoor foraging increased between the ages of 11 and 15 weeks for both slow- and fast-growing broiler strains, but acknowledged that broilers rarely consumed larvae or pupae based on an analysis of crop contents. Broilers of the current study were never observed foraging in dung pats for larvae and it seems reasonably unlikely that they would suddenly include this novel foraging technique to their behavioral repertoire as they approach slaughter weight at approximately 12 weeks of age. Yet, it is also possible for broilers to learn specialized foraging strategies depending on the social structure of the flock since domestic fowl engage in social learning during foraging events [286]. Therefore, an interaction between age and social learning of the flock may affect the success of foraging for fly larvae in dung pats for broilers.

To our knowledge, this is the first study to provide evidence that Freedom Ranger broilers do not forage for face fly larvae in cow dung pats in uncovered cattle pasture. Future research should investigate other poultry types and species, including laying hens, ducks, and geese, to fully understand whether poultry may be used as a biological control for managing dung flies on organic dairy farms.

5.6. Conclusions

Pastured Freedom Ranger hybrid broiler chickens stocked at 2.5 m^2 and 0.5 m^2 of outdoor area per broiler had no effect on the survival of face fly larvae in cow dung pats in this study. Larva survival rates were greater when reared in an environmentally controlled greenhouse compared to those reared on pasture. Solar radiation had a moderate to strong negative association with broiler pasture ranging. The comprehensive climate index (i.e., apparent temperature) was associated with broiler allocations of time spent sitting and sleeping, indicating that weather conditions may displace broiler time budgets. Broilers were never observed foraging in dung pats but were often observed foraging in other areas of the pen. Broiler pasture ranging and behavioral results indicate that weather conditions may affect the behaviors necessary for dung fly larva predation, but nevertheless pastured Freedom Ranger hybrid broiler chickens were not a successful method of face fly larva control in this study.

5.7. Publisher and Collaborator Recognition

A modified version of this chapter was accepted for publication in *Animals* on 2020 December 18 [287]. I would like to express my gratitude to Roger Moon, Ulrike Sorge and Bradley Heins for their contributions to this study and their co-authorships for this publication. I would also like to express gratitude to Darin Huot, summer interns and staff at West Central Research and Outreach Center for their assistance in data collection and care of animals. This work is supported by Organic Agriculture Research and Extension Initiative (OREI) [grant no. 2016-51300-25734/project accession no. 1010693] from the USDA National Institute of Food and Agriculture.

Chapter 6: Effects of Outdoor Stocking Density on Growth, Feather Damage and Behavior of Slow-Growing Free-Range Broilers

6.1. Summary

Access to pasture is a main benefit of free-range broiler housing systems, yet the impact of outdoor stocking density on broiler animal welfare remains unsettled. Growth, feather damage, pasture ranging and behaviors were assessed for 150 mixed-sex, slow-growing Freedom Rangers from 5 to 11 weeks of age with free access to either a high outdoor stocking density pasture (0.5 m^2 per bird) or a low outdoor stocking density pasture (2.5 m^2 per bird). The probability (mean, 95% CI) of tail feather damage was greater for the high-density (23.1%, 16.3 to 31.7%) compared to the low-density group (11.9%, 7.1 to 19.3%). The percent of observations resulting in sunbathing and aggressive attacks (i.e., pecking and fighting behaviors) were greater for the high-density (1.0%, 0.6 to 1.8% and 0.5%, 0.2 to 1.3%, respectively) compared to the low-density group (0.3%, 0.1 to 0.7% and 0.1%, 0.0 to 0.4%, respectively). Furthermore, an interaction between treatment and age indicated that birds in the high-density group displayed greater stretching (during weeks 7 to 10) and panting (during weeks 6 and 9). Results of this study suggest that additional outdoor pasture space may be positively associated with broiler welfare.

Keywords: outdoor stocking density, free-range, broilers, ranging, behavior, welfare

6.2. Introduction

Management factors, such as range enrichment provisions, have been explored as methods to improve the health and behaviors of free-range, meat-type chickens (i.e., broilers). For example, Fanatico et al. [164] reported that outdoor structural enrichments improved range utilization and decreased sitting behaviors in broilers. Dawkins et al. [163] similarly reported that broilers preferred to range in spaces which provided tree cover. Bosco et al. [288] found that olive trees and tall grass in the outdoor area encouraged broilers to range and ingest more pasture contents compared to an uncovered outdoor area. Jones et al. [175] also found that outdoor areas planted with sapling trees improved broiler ranging. These studies provide evidence that the quality of the outdoor area is important for free-range boilers; however, it is unclear whether simply providing additional outdoor space for ranging improves the welfare of broilers.

It is important to understand the impact that the amount of outdoor space (i.e., outdoor stocking density) has on broiler welfare since many animal welfare programs require certain outdoor space allowances for poultry in order to meet certification labels (e.g., “free-range” labels) [172]. In fact, the amount of outdoor area provided for birds is one of the major defining characteristics differentiating between levels of these labels. The topic of outdoor stocking density is also at the forefront of organic poultry policy change in the US since outdoor space requirements for organic poultry are currently undefined. Although the amount of pasture space allowance is a main feature of free-range poultry housing systems, the role that outdoor stocking density plays on poultry health and behavior is still not well understood.

The aim of this study was to compare the effects of two common levels of outdoor stocking densities on the growth, feather damage, pasture ranging and behaviors of free-range broilers from 5 to 11 weeks of age. The outdoor stocking densities chosen for this study were similar to the current standards for "free-range" and "pasture-raised" chickens under the Certified Humane program (Humane Farm Animal Care, Middleburg, VA) and the American Humane Certified program (American Humane, Washington, DC).

6.3. Materials and Methods

6.3.1. Animal Care and Housing

The University of Minnesota Institutional Animal Care and Use Committee approved all animal care and procedures specific to this experiment (protocol number 1607-33960A).

The experiment was conducted from July to October 2018 at the West Central Research and Outreach Center (Morris, MN) on organic pastureland that housed organic dairy cows (*Bos taurus* L.). Details on farm management and animal care are described by Phillips et al. [287] and are therefore only briefly described in this article.

6.3.2. Experimental Design

This study was a randomized complete block design with repeated measures to evaluate 150 Freedom Ranger (Welp Hatchery, Bancroft, IA) chickens (*Gallus gallus domesticus* L.) in 3 mixed-sex replicated groups of 50 who hatched on 29 May, 9 July and 16 July, respectively. At 4 weeks of age, birds in each replicate were leg-banded with numbered ZBands (Chicken Hill Poultry, Horseshoe Bend, ID) and randomly assigned to a pen corresponding to one of two outdoor stocking density treatment groups: 1) 0.5 m^2 of outdoor area per bird (high-density) or 2) 2.5 m^2 of outdoor area per bird (low-density). Treatments were balanced by sex and initial body weight. The assessment of sex at 4 weeks of age had an average accuracy of 90% and was therefore not perfectly balanced between high-density (females = 33, males = 44) and low-density (females = 41, males = 32) treatment groups. The average body weights (± SD) of females and males at 4 weeks of age were 0.86 ± 0.2 kg and 0.97 ± 0.2 kg, respectively; and the average body weights of birds in the high- and low-density treatment groups were 0.91 ± 0.2 kg and 0.92 ± 0.2 kg, respectively. Birds remained in their treatment groups for the remainder of their production cycle until they reached 12 weeks of age when they were slaughtered.

Treatment pens are displayed in a photograph in Figure 6.1. Each pen housed 25 birds that had access to 1.8 × 3.7 m of a floorless mobile shelter (Chicken Ranger Coops, Narvon, PA); thus, the covered shelter stocking density was 0.27 m^2 per bird. Birds were confined to the shelter at night but had free access to pasture corresponding to their stocking density treatment group during the day. Birds had ad libitum access to water from an 18.2-L poultry waterer (Item # PPF5, Miller Manufacturing, Eagan, MN) and granite grit from a round ground feeder (Item # PH-100, Stromberg's, Hackensack, MN). Fanatico et al. [164] reported that from 3 to 11 weeks of age free-range, mixed-sex Delaware broilers of a slow-growing genetic strain consumed an average of 138 g of concentrate per bird when feed was offered ad libitum. Furthermore, Rivera-Ferre et al. [178] calculated that a 10% restricted diet providing 115 g of concentrate per bird from 4 to 11 weeks of age was adequate for free-range broilers of a similar hybrid genetic strain (ISA) to the Freedom Ranger strain used in the present study. Based on this information, each bird received on average 141 g of concentrate (20% crude protein; Chick Starter AMP, Vita Plus Corporation, Madison, WI) daily prior to shelter confinement. For each pen, the feed was placed in a 121.2-cm long galvanized steel ground trough (Item # PH-118, Stromberg's, Hackensack, MN), which was removed and sanitized the following morning.

The mobile shelter and corresponding pens were relocated every 3 to 4 days to give birds at least 50% forage ground cover. Forage biomass and height in pens were measured using a rising plate meter (30 samples per pen; Jenquip, Feilding, New Zealand) prior to and after rotation to quantify the available and residual forage, respectively. The average ± SD forage biomass throughout the study was 1.8 ± 0.5 and 1.7 ± 0.3 Mg/ha for pens of the high- and low-density treatment groups, respectively. The average ± SD forage height measured over the course of the study was 9.1 ± 3.8 and 8.7 ± 2.2 cm for pens in the high- and low-density treatment groups, respectively. The orientation of the shelter alternated between facing either East or West approximately every 3 rotations.

Figure 6.1. View of high (left) and low (right) outdoor stocking density treatment pens for replicate 1 birds on 27 June at 08:55. At the time this photo was taken, the average forage biomass for high- and low-density pens was 2.6 and 1.6 Mg/ha, respectively; and the average forage height for high- and low-density pens was 15.2 and 7.5 cm, respectively.

6.3.3. Data Collection

Body weight and feather damage scores for each individual bird was assessed prior to study initiation, starting at 4 weeks of age and weekly thereafter. Feather damage scores for the back, thigh, tail and wing areas were conducted using a visual feather assessment: 0 = fully feathered, 1 = rough, 2 = some broken feathers and small bald areas, 3 = heavily broken feathers and some bald areas, 4 = almost bald or large bald areas and 5 = bald with no feather cover [289].

Behavior observations were recorded by a single observer 4 times per week in the morning (between 08:00 and 11:45) and afternoon (between 12:00

and 18:45) when there was no precipitation. The time range for observations was intended to encompass time points relative to daylight between 2 hours after sunrise and 2 hours prior to sunset. Noon (i.e., 12:00) was used to delineate between morning and afternoon time of day categories, therefore the time range of the afternoon was greater than the time range of the morning. Prior to behavior observations, pasture ranging was recorded for each pen as the number of birds outside of the shelter. Behaviors were then recorded in continuous 60-second observation periods on 10 individual focal birds per pen using the Animal Behaviour Pro mobile app (version 1.2) [265]. Focal birds were identified using livestock paint prior to study initiation and were observed in random order alternating between treatment pens. Behavioral states corresponding to the time budget were recorded as durations and behavioral events were recorded as binary outcomes (i.e., the occurrence of a behavioral event within the 60-second observation period was recorded as either a yes [presence] or no [absence]). An ethogram defining recorded behaviors is in Table 6.1.

Table 6.1. Ethogram for behaviors of mixed-sex Freedom Ranger chickens raised in a free-range system from 5 to 11 weeks of age observed in the range and shelter. Descriptions are modified from Ventura et al. [266], Mollenhorst et al. [290] and Santos et al. [291].

Behavior	Description
Behavioral states [1]	
Sitting	Bird has its breast in contact with the ground. Eyes are open
Standing	Bird maintains upright position on its extended, stationary legs
Sleeping	Bird has its breast in contact with the ground. Eyes are closed
Walking	Bird moves across the ground, wherein the legs propel the bird at a low speed
Running	Bird moves across the ground, wherein the legs propel the bird at a high speed
Behavioral events [2]	
Preening	Bird uses its beak to peck, stroke, or comb plumage
Foraging	Bird pecks or scratches at the ground
Stretching	Bird elongates its wing or its leg slowly
Grooming	Bird cleans, massages, or rubs itself using beak or feet
Disturbance	A bird makes physical contact with a resting bird, causing it to adjust or stand
Panting	Bird has beak open to respire
Drinking	Bird submerges beak into the water of the drinker
Flapping	Bird is in an upright position and extends its wings repeatedly
Sunbathing	Bird holds one or both wings out from the body with feathers spread
Dustbathing	Lying bird tosses dirt onto its back and wings by ruffling and shaking its body
Aggressive attack [3]	
Peck	Bird raises its head and strikes another bird with its beak
Fight	Two standing birds raise heads to face each other, one or both deliver > 2 kicks to opponent
Aggressive display [4]	
Threat	Bird stands with raised feathers and neck while opponent holds its head at lower level
Chase	A bird runs > 3 steps after another bird
Standoff	Two birds face each other with heads at same level for > 2 seconds
Leap	Two birds face each other, one or both jump without extending legs toward other bird

[1] Behavioral states are mutually exclusive. Recorded as duration. [2] Behavioral events are non-mutually exclusive. Behavioral events and states are non-mutually exclusive. Recorded as binary outcomes. [3] Observations in the categories peck and fight were analyzed as aggressive attack. [4] Observations in the categories threat, chase, standoff, and leap were analyzed as aggressive display.

The University of Minnesota West Central Research and Outreach Center weather station recorded ambient humidity, ambient temperature, precipitation, solar radiation, and wind speed every 15 minutes. The comprehensive climate index (CCI; i.e., apparent temperature) was calculated based on ambient humidity, ambient temperature, solar radiation and wind speed [267]. For each behavioral observation, the time was rounded to the nearest 15-minute and matched with the weather data.

6.3.4. Statistical Analysis

All analyses were performed in RStudio (version 1.3.1073) [194] with linear mixed models and mixed logistic regression models using the *glmmTMB* function [197]. For all models, fixed effects were *replicate* (3 levels), *treatment* (2 levels), *age* (7 levels), the *treatment* × *age* interaction and the random effect of *experimental unit* (pen; 6 levels). The first order autocovariance structure was used to account for repeated measures. Likelihood ratio tests (LRT) were used to assess the significance of fixed effects by comparing full and reduced models [241].

The analyses of body weight, feather damage and behaviors included a fixed effect of *sex* (2 levels) and a random effect of *bird identification* (ID). The analysis of body weight also included a continuous covariate for initial body weight recorded prior to treatment initiation when birds were 4 weeks of age. The interaction between treatment and sex for the analysis of body weight was initially tested but was removed from the model based on its insignificant effect.

Since recorded feather damage scores were no greater than 1, feather damage scores were dichotomized into no damage (0; score = 0) and damage (1; score ≥ 1) binary outcomes and the analyses were performed under a binomial error distribution. Pasture ranging and behavior outcomes were aggregated into weekly summations; ranging and behavioral states were analyzed with a beta-binomial error distribution and behavioral events were analyzed with a binomial error distribution. No birds were observed panting during weeks 10 and 11 of the study so these weeks were removed from the analysis. Data for rarely observed behavioral events (drinking, flapping, sunbathing, aggressive display, dustbathing, and aggressive attack) were pooled over weeks by obtaining a single summation for each focal bird and outcome. Behavioral events pooled across weeks did not include fixed or random effects containing age and did not include the random effect of bird ID.

Significance was declared when $p \leq 0.05$. The Tukey adjustment was applied for pairwise comparisons. Marginal means and 95% CI for feather damage, pasture ranging, and behaviors are reported as values back-transformed from the logit scale.

6.4. Results

6.4.1. Weather

Daily weather conditions while birds of all 3 replications were housed in mobile shelters (26 June to 8 October) over the course of the study are presented in Figure 6.2. The averages ± SD for ambient humidity, ambient temperature, solar radiation, wind speed and CCI during the study were 70 ± 9%, 20 ± 6 °C, 418 ± 174 W/m^2, 1.1 ± 0.4 m/second and 25 ± 8 °C, respectively. On average, morning observations had 76 ± 7% humidity, 18 ± 6 °C ambient temperature, 382 ± 188 W/m^2 solar radiation, 1.0 ± 0.4 m/second wind speed and 23 ± 8 °C CCI. Meanwhile, afternoon observations had 64 ± 8% humidity, 22 ± 6 °C ambient temperature, 454 ± 150 W/m^2 solar radiation, 1.1 ± 0.5 m/second wind speed and 27 ± 8 °C CCI, on average.

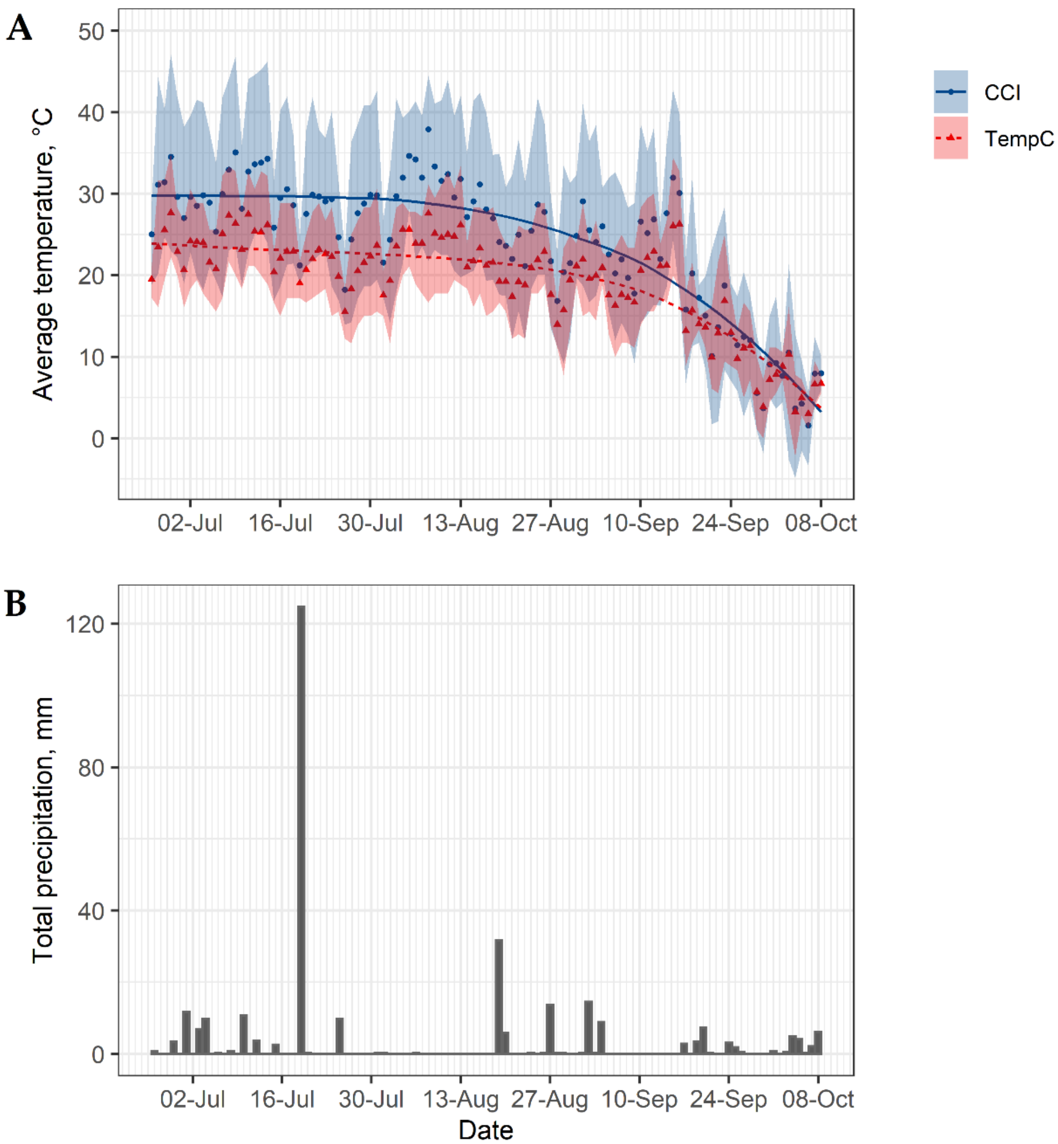

Figure 6.2. Daily weather during experiment. A) Average daily ambient temperature and average daily comprehensive climate index (CCI; i.e., apparent temperature). Lines are the best fit locally estimated scatterplot smoothing (loess) regressions. Transparent bands represent daily minimum and maximum values. B) Total daily precipitation.

6.4.2. Body Weight

Growth rates were similar among treatment groups, such that the mean body weights (95% CI) for broilers in the high- and low-density treatment groups were 2.2 kg (2.2 to 2.3 kg) and 2.2 kg (2.1 to 2.2 kg), respectively, when averaged across age. An effect of age indicated that birds became significantly heavier each week (Table 6.2). Birds gained between 0.2 and 0.4 kg per week. Furthermore, there was an effect of sex on body weight, such that mean body weight of males was greater than females (Table 6.3).

Table 6.2. Means ± 95% CI of animal-based indicators and behaviors affected by age for mixed-sex Freedom Ranger chickens raised in a free-range system from 5 to 11 weeks of age [1]

	Age (weeks)						
Outcomes [2]	5	6	7	8	9	10	11
	kg						
Body weight	1.1 [1.1, 1.2] [g]	1.5 [1.4, 1.5] [f]	1.9 [1.8, 1.9] [e]	2.3 [2.2, 2.3] [d]	2.5 [2.5, 2.6] [c]	3.0 [2.9, 3.0] [b]	3.2 [3.1, 3.2] [a]
	%						
Wing damage	23.9 [14, 38] [d]	55.8 [43, 68] [c]	73.2 [61, 83] [bc]	79.2 [67, 88] [b]	82.7 [72, 91] [b]	96.4 [91, 99] [a]	94.8 [89, 98] [a]
Tail damage	2.6 [0.7, 9.0] [b]	17.3 [10, 28] [a]	28.2 [19, 40] [a]	25.5 [17, 37] [a]	21.8 [14, 33] [a]	19.0 [11, 30] [a]	21.6 [13, 33] [a]
Ranging	34.2 [23, 47] [bc]	17.7 [11, 28] [c]	25.9 [17, 38] [bc]	36.2 [25, 49] [bc]	38.0 [27, 51] [bc]	44.6 [32, 58] [ab]	69.1 [52, 82] [a]
Sitting	55.0 [47, 62] [a]	52.3 [45, 60] [ab]	49.4 [42, 57] [ab]	45.6 [38, 53] [ab]	43.2 [36, 51] [b]	16.6 [12, 22] [c]	15.8 [11, 22] [c]
Standing	22.8 [17, 29] [bc]	21.4 [16, 28] [c]	15.2 [11, 20] [c]	23.8 [18, 30] [bc]	32.0 [25, 40] [b]	64.1 [55, 72] [a]	56.3 [47, 65] [a]
Sleeping	10.4 [8, 14] [cd]	22.6 [18, 28] [a]	26.5 [22, 32] [a]	18.5 [15, 23] [ab]	13.3 [10, 17] [bc]	7.1 [5, 10] [cd]	5.0 [3, 8] [d]
Walking	3.0 [2.2, 4.2] [ac]	1.7 [1.2, 2.5] [bd]	1.5 [1.0, 2.2] [d]	1.3 [0.9, 2.0] [d]	1.5 [1.0, 2.2] [d]	1.6 [1.0, 2.6] [cd]	3.4 [2.3, 5.1] [ab]
Running	0.2 [0.1, 0.4] [a]	0.1 [0.0, 0.2] [ab]	0.0 [0.0, 0.1] [b]	0.1 [0.0, 0.2] [ab]	0.1 [0.0, 0.2] [ab]	0.2 [0.1, 0.4] [a]	0.2 [0.1, 0.4] [a]
Preening	29.4 [25, 35] [ab]	30.1 [26, 34] [a]	24.9 [21, 29] [abc]	20.6 [17, 25] [bc]	25.9 [22, 30] [abc]	22.2 [17, 28] [abc]	16.1 [12, 22] [c]
Foraging	29.8 [25, 36] [a]	23.9 [20, 28] [a]	15.4 [12, 19] [b]	20.9 [17, 26] [ab]	15.8 [13, 20] [b]	24.6 [19, 31] [ab]	22.6 [17, 30] [ab]
Grooming	7.4 [5, 11] [a]	4.6 [3, 7] [ab]	2.8 [2, 5] [ab]	2.2 [1, 4] [b]	2.4 [1, 4] [b]	2.2 [1, 6] [ab]	2.3 [1, 7] [ab]
Disturbance	6.3 [4, 10] [a]	3.5 [2, 6] [ab]	2.0 [1, 4] [b]	2.3 [1, 5] [ab]	1.0 [0.4, 2] [b]	1.0 [0.2, 4] [ab]	3.4 [1, 8] [ab]

[a–g] means within a row with different letter superscripts are different after Tukey's adjustment, $p \leq 0.05$. [1] Body weight ($X^2_{(6)} = 1872.7$), wing damage ($X^2_{(6)} = 139.1$), tail damage ($X^2_{(6)} = 29.4$), ranging ($X^2_{(6)} = 22.1$), sitting ($X^2_{(6)} = 128.1$), standing ($X^2_{(6)} = 134.0$), sleeping ($X^2_{(6)} = 89.4$), walking ($X^2_{(6)} = 29.4$), running ($X^2_{(6)} = 27.7$), preening ($X^2_{(6)} = 24.9$), foraging ($X^2_{(6)} = 29.5$), grooming ($X^2_{(6)} = 18.1$) and disturbance ($X^2_{(6)} = 29.8$) were affected by age ($p < 0.01$). Behaviors of drinking, flapping, sunbathing, aggressive display, dustbathing, and aggressive attack were pooled over age. Stretching ($X^2_{(6)} = 18.5$) and panting ($X^2_{(6)} = 16.6$) were affected by the treatment × age interaction ($p < 0.01$).
[2] Results are reported as probabilities for feather damage, % of birds for ranging, % of time for sitting, standing, sleeping, walking, and running, and % of observations for preening, foraging, grooming and disturbance.

Table 6.3. Means ± 95% CI of animal-based indicators and behaviors affected by sex for mixed-sex Freedom Ranger chickens raised in a free-range system from 5 to 11 weeks of age [1]

	Sex		Sex effect [2]	
Outcome	Male	Female	$X^2_{(1)}$	p
Body weight, kg	2.3 [2.3, 2.4]	2.1 [2.0, 2.1]	73.3	<0.01
Wing feather damage, % probability	68.0 [57.5, 77.0]	86.6 [78.8, 91.9]	16.6	<0.01
Preening, % observations	20.9 [18.8, 23.3]	27.0 [24.5, 29.7]	13.2	<0.01
Panting, % observations	1.2 [0.7, 2.2]	2.0 [1.2, 3.4]	3.8	0.05
Aggressive display, % observations	1.0 [0.3, 2.7]	0.1 [0.0, 0.6]	13.3	<0.01
Aggressive attack, % observations	0.5 [0.2, 1.2]	0.1 [0.0, 0.5]	5.4	0.02

[1] Tail feather damage ($X^2_{(1)} = 0.5$, $p = 0.47$), sitting ($X^2_{(1)} = 0.5$, $p = 0.50$), standing ($X^2_{(1)} = 0.1$, $p = 0.70$), sleeping ($X^2_{(1)} = 2.1$, $p = 0.14$), walking ($X^2_{(1)} = 0.2$, $p = 0.67$), running ($X^2_{(1)} = 0.0$, $p = 0.99$), foraging ($X^2_{(1)} = 1.0$, $p = 0.31$), stretching ($X^2_{(1)} = 0.1$, $p = 0.80$), grooming ($X^2_{(1)} = 1.5$, $p = 0.23$), disturbance ($X^2_{(1)} = 0.4$, $p = 0.51$), drinking ($X^2_{(1)} = 0.0$, $p = 0.82$), flapping ($X^2_{(1)} = 0.7$, $p = 0.39$), sunbathing ($X^2_{(1)} = 0.1$, $p = 0.81$) and dustbathing ($X^2_{(1)} = 0.2$, $p = 0.67$) were not affected by sex.
[2] Chi-square statistic of likelihood ratio test (LRT).

6.4.3. Feather Damage

There was an effect of stocking density treatment on tail feather damage (Table 6.4). Broilers in the high-density group had greater tail feather damage compared to broilers in the low-density group. Yet, birds had similar wing feather damage over the course of the study regardless of treatment. There was an effect of age on wing and tail feather damage (Table 6.2). In general, the probability of observing wing feather damage increased as birds aged, and tail feather damage was the lowest at 5 weeks of age compared to all other weeks. There was an effect of sex on wing feather damage, in which females had a greater probability for wing feather damage compared to males (Table 6.3). Neither back nor thigh feather damage was observed for any birds during the study.

Table 6.4. Means ± 95% CI of animal-based indicators and behaviors affected by outdoor stocking density treatment (high: 0.5 m^2 of pasture per bird; low: 2.5 m^2 of pasture per bird) for mixed-sex Freedom Ranger chickens raised in a free-range system from 5 to 11 weeks of age [1]

	Treatment		Treatment effect [2]	
Outcomes	High	Low	$X^2_{(1)}$	p
Tail feather damage, % probability	23.1 [16.3, 31.7]	11.9 [7.1, 19.3]	6.2	0.01
Sunbathing, % observations	1.0 [0.6, 1.8]	0.3 [0.1, 0.7]	5.1	0.02
Aggressive attack, % observations	0.5 [0.2, 1.3]	0.1 [0.0, 0.4]	6.9	<0.01

[1] Body weight ($X^2_{(1)} = 1.5$, $p = 0.22$), wing feather damage ($X^2_{(1)} = 1.0$, $p = 0.32$), ranging ($X^2_{(1)} = 1.1$, $p = 0.28$), sitting ($X^2_{(1)} = 1.0$, $p = 0.32$), standing ($X^2_{(1)} = 0.7$, $p = 0.40$), sleeping ($X^2_{(1)} = 0.2$, $p = 0.68$), walking ($X^2_{(1)} = 0.2$, $p = 0.64$), running ($X^2_{(1)} = 0.0$, $p = 0.99$), preening ($X^2_{(1)} = 0.1$, $p = 0.72$), foraging ($X^2_{(1)} = 3.0$, $p = 0.08$), grooming ($X^2_{(1)} = 0.3$, $p = 0.60$), disturbance ($X^2_{(1)} = 1.6$, $p = 0.21$), panting ($X^2_{(1)} = 2.4$, $p = 0.12$), drinking ($X^2_{(1)} = 2.0$, $p = 0.16$), flapping ($X^2_{(1)} = 0.0$, $p = 0.86$), aggressive display ($X^2_{(1)} = 0.0$, $p = 0.83$) and dustbathing ($X^2_{(1)} = 0.1$, $p = 0.82$) were not affected by treatment. Stretching ($X^2_{(6)} = 18.5$, $p < 0.01$) and panting ($X^2_{(6)} = 16.6$, $p < 0.01$) were affected by the treatment × age interaction effect. [2] Chi-square statistic of likelihood ratio test (LRT).

6.4.4. Behaviors

There was no effect of treatment on pasture ranging, such that a similar percentage of birds were observed pasture ranging between high-density (32.7%, 95% CI = 24.2 to 42.5%) and low-density (41.6%, 95% CI = 32.0 to 52.0%) groups. There was an effect of age (Table 6.2) and time of day (Table 6.5) on pasture ranging. In general, ranging increased with age and more birds were observed ranging in the morning compared to the afternoon.

Table 6.5. Means ± 95% CI of behaviors affected by time of day for mixed-sex Freedom Ranger chickens raised in a free-range system from 5 to 11 weeks of age [1]

	Time of day		**Time of day effect [2]**	
Behaviors	Morning	Afternoon	$X^2_{(1)}$	p
Ranging, % birds	47.1 [38.6, 55.7]	28.0 [21.5, 35.6]	18.6	<0.01
Behavioral state, % time				
Sitting	31.6 [26.6, 37.2]	44.5 [38.6, 50.7]	46.8	<0.01
Standing	39.9 [34.0, 46.1]	24.8 [20.2, 30.0]	61.2	<0.01
Sleeping	11.3 [9.6, 13.2]	15.1 [13.0, 17.4]	11.0	<0.01
Walking	2.2 [1.7, 2.9]	1.6 [1.2, 2.1]	10.0	<0.01
Running	0.1 [0.1, 0.2]	0.1 [0.0, 0.1]	10.8	<0.01
Behavioral event, % observations				
Foraging	24.8 [22.0, 27.9]	18.5 [16.0, 21.2]	15.0	<0.01
Stretching	2.6 [1.8, 3.7]	4.6 [3.4, 6.3]	11.0	<0.01
Panting	0.7 [0.4, 1.2]	3.7 [2.4, 5.9]	75.6	<0.01
Flapping	0.3 [0.1, 0.8]	1.0 [0.5, 1.7]	4.8	0.03
Aggressive attack	0.4 [0.2, 1.2]	0.1 [0.0, 0.5]	5.6	0.02

[1] Preening ($X^2_{(1)}$ = 1.7, p = 0.19), grooming ($X^2_{(1)}$ = 0.0, p = 0.98), disturbance ($X^2_{(1)}$ = 0.1, p = 0.79), drinking ($X^2_{(1)}$ = 0.9, p = 0.34), sunbathing ($X^2_{(1)}$ = 0.6, p = 0.44) aggressive display ($X^2_{(1)}$ = 1.0, p = 0.32) and dustbathing ($X^2_{(1)}$ = 0.9, p = 0.33) were not affected by time of day. [2] Chi-square statistic of likelihood ratio test (LRT).

Behavioral states of the time budget were similar among treatment groups and sex. Sitting, standing, and sleeping were the most commonly observed behavioral states, followed by walking and running. There was an effect of age (Table 6.2) and time of day (Table 6.5) on all behaviors of the time budget. Older birds generally had a more active time budget, in which sitting decreased and standing increased with age. Sleeping increased until 7 weeks and decreased thereafter. Walking decreased until weeks 7 to 9 and then increased thereafter. Running was greatest at weeks 5, 10 and 11 compared to week 7. A more active time budget was observed in the morning compared to the afternoon, such that more time was spend standing, walking, and running during the morning and more time was spent sitting and sleeping during the afternoon.

The behavioral events in order from greatest to least commonly recorded were: preening, foraging, stretching, grooming, disturbing, panting, drinking, flapping, sunbathing, aggressive display, dustbathing and aggressive attack. For the aggressive display category, threats and chases were most common, followed by standoffs and leaps. For the aggressive attack category, pecking was more commonly observed than fighting. There were no effects of treatment, age, sex nor time of day on drinking and dustbathing; the overall mean percentage (95% CI) of observations recorded for these behavioral events were 1.9% (1.5 to 2.6%) and 0.3% (0.1 to 0.8%), respectively.

There was an effect of stocking density treatment on two behavioral events (Table 6.4). Birds in the high-density group were more commonly observed sunbathing and performing aggressive attacks compared to birds in the low-density group. There was also a trend ($p = 0.08$) for the effect of treatment on foraging, such that the mean percent (95% CI) of observations in which foraging was recorded was 23.8% (20.7 to 27.3%) for birds in the low-density group and 19.3% (16.5 to 22.4%) for birds in the high-density group.

There was an interaction present between treatment and age for stretching (Figure 6.3). Birds in the high-density group showed greater stretching during weeks 8, 9 and 10 compared to birds in the low-density group ($p \leq 0.03$). For birds in the low-density group, stretching was greater during week 6 compared to week 9 ($p = 0.03$) while the remaining weeks were similar. Stretching events were similar between all weeks for birds in the high-density group ($p > 0.94$).

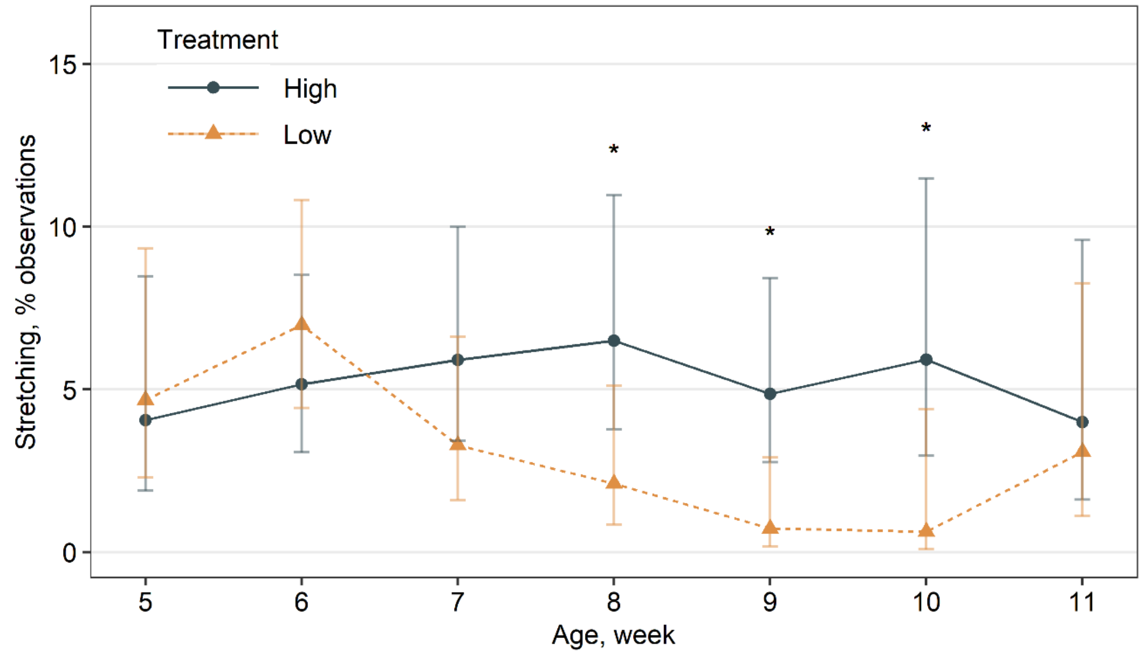

Figure 6.3. Means ± 95 CI of stretching events for outdoor stocking density treatment (high: 0.5 m^2 of pasture per bird; low: 2.5 m^2 of pasture per bird) and age interaction effect. * means within a week are different after Tukey's adjustment, $p \leq 0.03$.

There was an interaction present between treatment and age for panting (Figure 6.4). No focal birds were observed panting during weeks 10 and 11 of age. Birds in the high-density group showed greater panting during weeks 6 and 9 compared to birds in the low-density group ($p \leq 0.03$). For birds in the low-density group, panting was greater during week 6 compared to weeks 5, 10 and 11 ($p < 0.01$) while the remaining weeks were similar. For the high-density group, panting was highest during weeks 6 and 9 compared to all other weeks ($p \leq 0.01$).

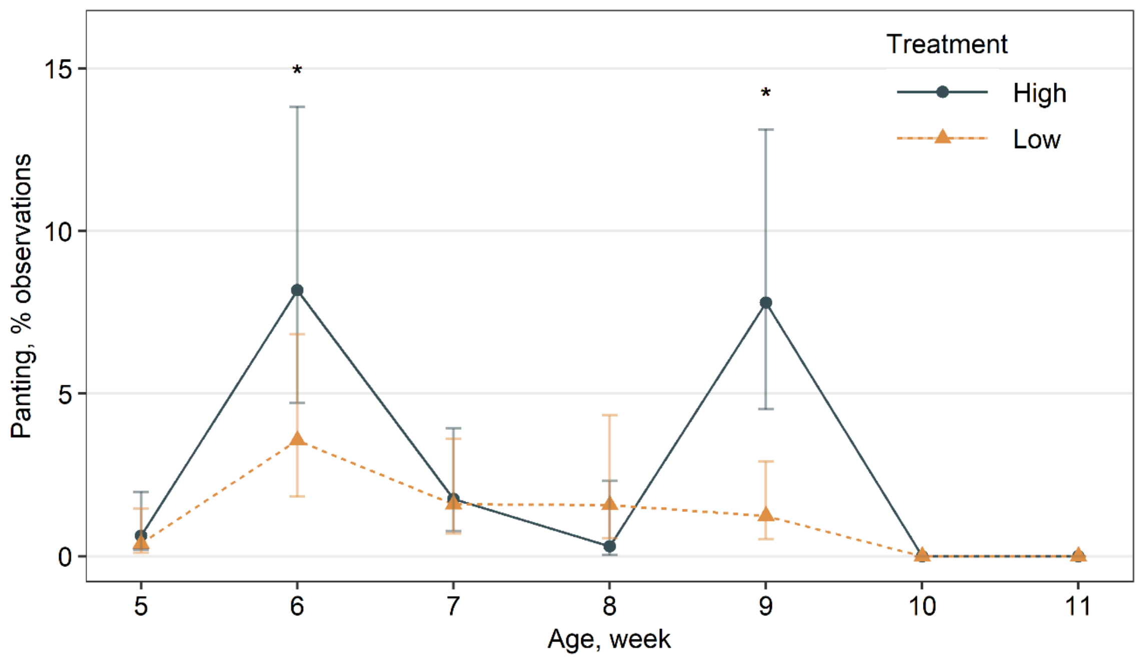

Figure 6.4. Means ± 95 CI of panting events for outdoor stocking density treatment (high: 0.5 m^2 of pasture per bird; low: 2.5 m^2 of pasture per bird) and age interaction effect. No birds were observed panting during weeks 10 and 11 so these weeks were removed from the analysis. * means within a week are different after Tukey's adjustment, $p \leq 0.01$.

There was an effect of age on preening, foraging, grooming and disturbance events (Table 6.2). The remaining behavioral events (except stretching and panting) were pooled over weeks and therefore the effect of age could not be assessed. Preening generally decreased with age. Foraging was highest during week 5 and lowest during weeks 7 and 9. In general, foraging was quite variable across age. Grooming and disturbance behaviors decreased with age.

There was an effect of sex on preening, panting, aggressive display, and aggressive attack (Table 6.3). Preening and panting were more commonly observed for females, while aggressive displays and attacks were more commonly observed for males.

There was an effect of time of day on foraging, stretching, panting, flapping and aggressive attack events (Table 6.5). Foraging and aggressive attacks were more commonly observed in the morning, while stretching, panting, and flapping were more commonly observed in the afternoon.

6.5. Discussion

6.5.1. Effects on Growth and Activity

There were no effects of treatment on body weight nor behavioral states in feed-restricted broilers, suggesting that growth and activity levels were not affected by outdoor stocking density. Other studies similarly reported that outdoor enrichment provisions did not affect body weight growth of free-range broilers [164,288]. Another study by Jones et al. [175] also found no effect of outdoor stocking density (1.2 *vs* 2.5 m^2 per bird) on free-range broiler growth, pasture ranging nor behaviors (i.e., drinking, foraging, lying, sleeping, standing and walking).

Body weight could conceivably be affected by stocking density if the activity or stress levels of birds are altered. For example, Sanchez-Casanova et al. [17] found that broilers raised indoors at a low stocking density (0.2 m^2 per bird) had an increased growth, whereas broilers raised with outdoor access had decreased growth which could be partially explained by elevated activity.

6.5.2. Effects on Feather Damage and Aggression

Tail feather damage and aggressive attacks (i.e., pecking and fighting) were greater for birds reared at a high outdoor stocking density compared to a low outdoor stocking density. Gocsik et al. [292] similarly reported that plumage cleanliness was improved for broilers with a lower outdoor stocking density (1 *vs* 4 m^2 per bird). Nicol et al. [293] also reported increased feather damage and pecking behaviors as stocking density increased; however, this study investigated laying hens at indoor stocking densities (from 0.03 to 0.17 m^2 per bird) much higher than those of the current study. On the contrary, Huo and Na-Lampang [177] reported no effects of indoor stocking density (from 0.06 to 0.13 m^2 per bird) on aggressive attack behaviors nor feather damage in Thai crossbred broilers from 4 to 12 weeks of age.

Increased tail feather damage in birds of the high outdoor stocking density group may be partially explained by elevated aggressive attacks. An aggressive attack was a result of physical conflict between birds, such as pecking or fighting. These physical altercations were mostly comprised of pecking while fighting was rarely observed. Pecking behaviors have been previously demonstrated as a result of competition for a limited resource, such as food [294]. Although birds of the present study were feed-restricted, the amount provided was greater than amounts used in previous studies [164,178]. Alternatively, it is possible that the physical aggressive attacks were an attempt to form a social hierarchy, as suggested by Rushen [295] who reported that dominance relationships formed around 4 to 5 weeks of age in Rhode Island Red × White Leghorn pullets.

The effects of stocking density on aggression in free-range broilers is not a well understood topic. Introducing a complex environment, such as pasture access, may shift the behavioral dynamics of poultry [17,296]. Fanatico et al. [164] reported that aggressive behaviors were more likely to occur outdoors than indoors for free-range broilers. It is possible that birds in the low outdoor stocking density group were able to evade escalading aggressive conflicts by temporarily dispersing among the pasture. Meanwhile, birds in the high outdoor stocking density group may have been incapable of avoiding conflict given the limited availability of pasture space, resulting in increased physical aggressive attacks with other birds.

The occurrence of aggression was lower than reported for previous studies. For the current study, at least one of the 6 recorded aggression events (i.e., peck, threat, chase, standoff, fight, or leap) was observed in 1.3% of observations. Fanatico et al. [164] reported that aggression events for broilers reared with access to pasture were observed in 5.4% of observations when averaged across pen location and age; however, disturbance was also categorized as aggression in this study. Regardless, aggressive displays and aggressive attacks were rarely observed compared to all other behavioral events recorded for birds of the current study.

6.5.3. Effects on Pasture Ranging

It is not surprising that outdoor stocking density did not affect pasture ranging. Previous studies demonstrated that broilers rarely venture further than the immediate vicinity of the shelter, even when provided covered areas in the outdoor space [163,164,166,178]. Although birds in the low outdoor stocking density group had more pasture space available, it is likely that they remained in close proximity to the shelter.

The average percentage of birds outside of the shelter was 37%, when aggregated across all effects. This finding is similar to Stadig et al. [166] who reported an average of 40% of broilers observed pasture ranging. However, findings for pasture ranging were greater than several previous studies, which reported that 5 to 15% of broilers were observed outside their shelters on average [163,164,175,297]. The high level of pasture ranging for the current study may have been due to differences in bird genetics and weather conditions between studies [162,166,298]. The high use of the range in the morning is in agreement with other studies [162–164,175,299].

6.5.4. Effects on Behaviors

In addition to aggressive attacks, outdoor stocking density also had an effect on stretching, panting and sunbathing behavioral events. Birds in the high outdoor stocking density group were more commonly observed stretching (during weeks 7 to 10), panting (during weeks 6 and 9) and sunbathing. In a study of broilers reared in a free-range system, Gonçalves et al. [15] reported that behaviors defined as “movements to stretch the wings and legs on the same side of the body simultaneously, shaking and whirring feathers, lifting part of both wings close to the body or extend the tips of the wings and/or flapping it” were more evident for fast-growing genetic strains with greater body weights and at higher temperatures, indicating that these types of behavioral adjustments may have been used to cope with discomfort, especially heat stress. Furthermore, previous studies [291,300] showed that broilers will pant as method to cope with air temperatures above their thermal neutral zone. This information possibly indicates that birds of the high outdoor stocking density experienced greater discomfort from heat compared to birds of the low outdoor stocking density group as indicated by elevated stretching and panting.

The increased sunbathing observed in broilers of the high outdoor density treatment group may also be related to high temperatures. Duncan et al. [301] suggest that radiant heat and light may trigger sunbathing in hens, which can shift to dustbathing if environmental factors are present, such as dry soil. Sunbathing and dustbathing have several shared body postures, such as side lying and feather spreading, which makes it convenient for a sunbathing bird to proceed with dustbathing. Yet, sunbathing is not a well understood behavior in domesticated poultry species and is therefore challenging to deduce the motivation for birds in the high outdoor stocking density group to perform this behavior.

The explanation for heat stress in birds of the high outdoor stocking density group remains obscured. Even though treatment groups had a similar quantity of shade from the covered shelter, it is possible that birds in the high outdoor stocking density group experienced restricted options for shade from vegetative cover, whereas birds in the low outdoor stocking density group may have had an increased opportunity to seek shade in forages due to the greater amount of space they were provided. Dense, tall stands of vegetation could theoretically provide adequate shade for free-range broilers. For example, Jones et al. [175] reported that sapling trees with a mean height of 83 cm encouraged broilers to use the range on sunny days, indicating that the vegetation in this study may have provided some relief from solar intensity in the range. This study [175] also found that broilers were more likely pant inside their shelter compared to in their range, suggesting that birds were able to alleviate some heat stress by seeking relief in the range.

Although the maximum average forage height in pens was only 23 cm during the current study, there was significant variation in forage height and density within pens that created a diverse habitat. Dawkins et al. [163] used preference testing to demonstrate that free-range broilers actively selected their habitat within the outdoor space provided to them, wherein birds chose habitats occupied by trees, bushes, hedge or long grass. Furthermore, tunneling behaviors in tall grasses have been documented in free-range broilers [302], which may use this adapted behavior as a method to self-regulate body temperature. Although behavioral interactions with vegetation in the outdoor area were not intentionally recorded and analyzed, birds of the current study were observed tunneling in forages and commonly used the tunnels as a place to rest. Based on this information, it is possible that the low outdoor stocking density used for this study provided birds an opportunity to seek and select a suitable habitat within their range given that they had more outdoor space than birds in the high outdoor stocking density group.

It is unclear why panting was not observed during weeks 10 and 11 of the study. Although other heat-induced behaviors were not recorded for this study, it is possible that older birds learned to cope with heat stressors by using different strategies other than panting, such as opening their wings to dissipate heat [291,303]. However, a more probable explanation is that panting was not induced due to lower air temperature during this period. Santos et al. [291] reported that panting occurred in 4 to 6 week old naked neck broilers once the average air temperature reached approximately 34 °C. For the current study, the observations for weeks 10 and 11 occurred between 17 September and 1 October, in which the maximum air temperature only reached 25 °C (Figure 6.2). Based on this information, it is likely that panting was not observed during weeks 10 and 11 of the study due to cooler temperatures.

Observed behaviors were modified according to time of day, which may have been due to heat stress. For the current study, sitting, sleeping, stretching, panting and flapping events were mostly observed in the afternoon when temperatures were higher, indicating that these behaviors may have been attributed to coping with heat stressors. Gonçalves et al. [15] similarly reported greater sitting, stretching and flapping and a reduction in foraging and aggressive attacks (i.e., pecking) in the afternoon. Furthermore, previous studies [291,303] also reported that broilers were more likely to exhibit panting in the afternoon. The adaption of behaviors throughout the day may have been a result of heat stress and conservation of energy in the afternoon [291,294].

The behaviors exhibited by birds was modified according to age, yet the behavioral repertoire remained diverse throughout the study, which is in agreement with previous studies that investigated the behaviors of free-range broilers. Previous studies [32,297] similarly found that pasture ranging increased with age, which may be attributed to the familiarization of the outdoor environment to birds over time. Likewise, previous research [164,304] also reported a generally more active time budget as broilers aged. A study by Gonçalves et al. [15] reported that preening, foraging and stretching (combined with flapping) were the most commonly observed behavioral events in free-range broilers, while aggression and dustbathing were rarely observed. Fanatico et al. [164] reported that foraging was the most observed behavioral event in free-range broilers, followed by drinking, preening and dustbathing. In general, the behaviors examined in the present study demonstrated a wide range of activities that free-range slow-growing broilers partook in.

6.5.5. Limitations

There is no clear indication that outdoor stocking density to be a major influencer of broiler welfare at the levels investigated in this study. It is likely that stocking density, regardless of whether it is indoor or outdoor, is less important than the condition of the space provided, as suggested by Dawkins et al. [305]. Hence, the results of this study are most useful when applied to production systems of similar conditions, wherein the range consists of forages with varying heights and densities, is uncovered and does not provide outdoor enrichment. Other management factors such as indoor stocking density may also play an important role in the effects of varying levels of outdoor stocking density on the welfare of broilers. A previous study reported that broiler behavior depended on both outdoor access and indoor stocking density [17], suggesting that a different covered shelter stocking density than the one used in the present study may yield different results. Furthermore, recent research suggests that future studies on free-range broiler welfare should include detailed documentation on pasture use, such as number of visits and distances traveled, as it may help predict the welfare of broilers in free-range systems [299]. Authors of the current study also suggest that future studies should perhaps investigate the complex interactions birds have with their environment such as sunbathing, tunneling and other forage manipulation behaviors, as these behaviors are presently not well understood but are presumably fundamental behaviors in free-range systems.

6.6. Conclusions

Assessing the performance and behaviors of free-range broilers from 5 to 11 weeks of age provided evidence that additional outdoor pasture space may be positively associated with broiler welfare, including reduced tail feather damage, aggressive attacks and behaviors akin to discomfort such as stretching, panting and sunbathing. Furthermore, these findings also demonstrate the extensive array of species-specific behaviors of broilers raised in a free-range system. Results from this study suggest that the level of outdoor stocking density may play a role in improving free-range broiler welfare.

6.7. Publisher and Collaborator Recognition

A modified version of this chapter was accepted for publication in *Animals* on 2020 March 4 [306]. I would like to express my gratitude to Bradley Heins for his contributions to this study and his co-authorship for this publication. I would also like to express gratitude to summer interns and staff at the West Central Research and Outreach Center for their assistance in data collection and care of animals. This work is supported by Organic Agriculture Research and Extension Initiative (OREI) [grant no. 2016-51300-25734/project accession no. 1010693] from the USDA National Institute of Food and Agriculture.

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